

**THE DYNAMICS AND ROLE OF NEUTROPHILS IN AN
ELASTASE MODEL OF EMPHYSEMA**

by

Catherine Clare Bradley

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Abstract

Neutrophils are often early responders to pulmonary damage, rapidly infiltrating the lungs to promote tissue repair following exposure to harmful stimuli. A single dose of intratracheally (IT)-administered elastase results in acute inflammation and progressive emphysematous damage that is also accompanied by a transient and robust neutrophil influx. However, the importance of this early neutrophil recruitment in the chronic progression of emphysema is not well understood. This work seeks to better define the dynamics of pulmonary neutrophil migration in the elastase model of emphysema and to address the hypothesis that the early influx of neutrophils into the lungs contributes to the resolution of acute elastase-mediated injury and affords protection to the lungs from the progressive alveolar destruction that is associated with emphysema.

Bronchoalveolar lavage (BAL) samples were examined by light microscopy at acute time points post-porcine pancreatic elastase administration (post-PPE) to map the cellular response to three enzymatic units of IT elastase in neutrophil-intact BALB/c mice. Systemic depletion of neutrophils was accomplished using a single 500 µg dose of anti-mouse Ly-6G antibody (clone 1A8) injected intraperitoneally (IP) 24 hours prior to elastase treatment. BAL cell counts, total lung capacity (TLC), diffusion factor for carbon monoxide (DFCO), and mean airspace chord lengths (L_m) were each evaluated for effects of neutrophil depletion.

Neutrophils in BAL fluid from control animals were found to peak approximately 24 hours post-PPE before returning to baseline by 72 hours. Treatment with 1A8

resulted in sustained depletion of neutrophils for approximately 5 days that, as expected, ablated the early influx of neutrophils following PPE administration. Neutrophil-depleted mice were found to have no significant exacerbation of emphysematous damage post-PPE. These results do not support the hypothesis that neutrophils contribute to the resolution of lung injury.

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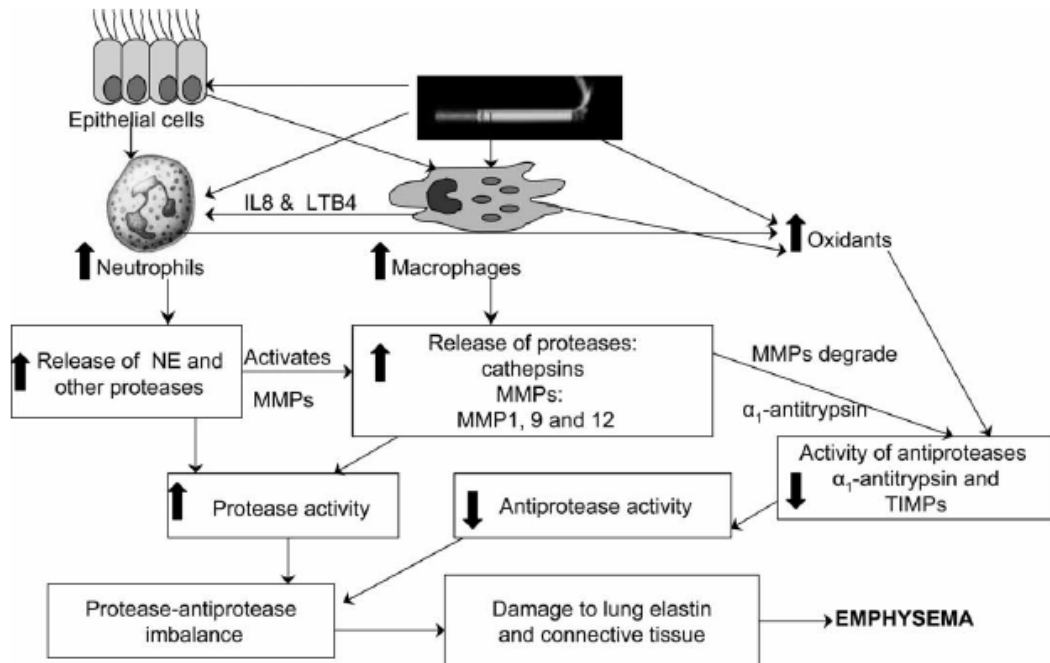
INTRODUCTION

Emphysema

Emphysema is characterized by the progressive destruction of alveoli accompanied by a reduction in ventilation and gas exchange (Mayo Clinic). It is grouped together with chronic bronchitis under a blanket of symptoms referred to as chronic obstructive pulmonary disease (COPD). Histologically, an emphysematous lung has wide air spaces, few dividing septa, and lacks the grape-like structure that lends a healthy lung its high surface area for efficient gas exchange. Emphysema patients are often short of breath, lethargic, and have a rapid pulse. These symptoms result because too little oxygen is able to diffuse across alveoli into pulmonary vessels to meet the body's demands. COPD patients are also at greater risk for airway infections. Whether these infections contribute to the disease or result from it is unclear. Environmental factors such as smoke, pollution, fumes, and dust can contribute to the development of emphysema. Emphysema does not appear to result from a single exposure. Rather, repeat exposures over years and years seems to drive disease. Habitual smokers comprise the vast majority of cases. But second-hand smoke and occupational exposures contribute to the prevalence of emphysema as well. Genetics is believed to be a contributing factor, but with the exception of a heritable deficiency in alpha 1-antitrypsin the mechanism is unclear. Evidence supporting the role of genetics includes habitual smokers who never develop emphysema despite extensive exposure to a known risk factor.

The protease-antiprotease hypothesis, depicted in Figure 1 (1), is the oldest and most studied mechanism for emphysema. This hypothesis posits a scenario in which lung damage drives the infiltration of neutrophils that release copious amounts of extracellular matrix-degrading enzymes, such as elastase. This elastase spike overwhelms the neutralizing power of local antiprotease molecules and extensive degradation of the lung parenchyma results.

Figure 1: A cigarette smoke-induced mechanism of the protease-antiprotease hypothesis.



Neutrophil expressed elastase (ELANE), IL-8 (the neutrophil chemotactic chemokine CXCL8), and MMP9 (matrix metalloproteinase 9; type IV collagenase; macrophage gelatinase) were all found to be elevated in COPD patient sputum and correlate with disease severity (2). The marked presence of matrix metalloproteinases and neutrophil elastase in smoker lungs is established, that they play a role in alveolar

destruction is very likely, but that neutrophils are the primary source has yet to be determined (3). Macrophages can secrete neutrophil elastase taken up from neutrophils (4). Matrix metalloproteinase 12 (MMP12), produced by macrophages, could deactivate the principle antiprotease responsible for neutralizing neutrophil elastase (3). Cigarette smoke itself is enough to simultaneously induce elastase and reduce the activity of its inhibitors (3). Rather than incriminate neutrophils, the protease-antiprotease hypothesis demonstrates the important role of the innate immune response in, at the very least, initiating emphysema. The elastin and collagenous extracellular matrix that forms the scaffold on which epithelial cells assemble into the unique grape-like clusters is directly destroyed by elastase. While this widely accepted hypothesis offers a mechanism that explains some aspects of alveolar destruction, it cannot account for all characteristics of the disease.

The disease outcomes for patients with emphysema are poor. While therapies do exist to slow the progression of emphysema and manage symptoms, the damage is lasting and irreversible. Doctors can prescribe supplemental oxygen, steroids, and/or bronchodilators; but ultimately the best option for patients is to quit smoking and limit their exposure to known irritants.

Emphysema costs the United States nearly 30 billion dollars annually (5). Approximately 4.7 million Americans – mostly adults above the age of 45 – suffer from emphysema, live a lower quality of life and will require more and more care as their disease progresses (5). On a global scale, emphysema – as measured by COPD estimates – affects 210 million people (6). Clearly this is a public health problem of enormous

reach. Given the poor prognosis that accompanies an emphysema diagnosis, the strategy should focus on disease prevention. This includes education, risk management, and research.

Little is understood of *how* factors such as cigarette smoke induce such dramatic and progressive pathology. The extent to which immune-mediated damage contributes to the disease has only been superficially characterized. The research detailed in this thesis is intended to add to this body of knowledge. Specifically, we seek to either absolve or incriminate polymorphonuclear leukocytes of a role in the development of emphysema.

Immunology of Emphysema

Emphysema is a chronic condition of sterile alveolar inflammation. There is no evidence that pathogens initiate or promote the destruction of alveolar ultrastructure in smoke-induced emphysema, but that does not absolve infectious agents of a potential role. The hookworm lifecycle has an obligatory lung phase and infection with hookworm parasites can initiate emphysematous changes (7). Retamales and colleagues published in 2001 that a greater degree of emphysematous pathology correlated with higher levels of adenovirus envelope protein expression (8). However, existing pathology could predispose a COPD patient to adenovirus infection. That the original pathology was caused by infection is not necessarily the case. On a similar note, macrolide research indicates this class of antibiotics is useful in decreasing the severity of COPD (9). Macrolides work by inhibiting protein synthesis and are considered

effective broad-spectrum antibiotics, but recent findings suggest certain macrolides also have anti-inflammatory properties. Deciphering which macrolide property – antimicrobial or immunomodulatory – is responsible for its therapeutic effects in COPD management is of greater interest. Perhaps it is a combination of both. There is evidence that products of the innate immune system – initially released into the lung to respond to cellular damage – actually contribute to airway damage (4). A better understanding of emphysema immunology could lead to earlier and more accurate diagnosis, effective treatments, and novel prevention strategies. If specific cells, cytokines, and activation states are found to correlate with emphysema, assays that detect these could diagnose a patient long before physiological symptoms become manifest. If specific cell types or immune mediators are discovered to contribute directly to disease, these could serve as targets for neutralizing antibodies or inhibitors. There is incredible potential for emphysema immunology research to translate to a clinical setting and benefit millions of people.

In emphysema, whether the insult is chemical – for example toxins found in tobacco smoke – or particulate – such as asbestos – the epithelial cells lining the lung are damaged. *How* epithelial cells become damaged is a more contentious topic. There are two likely mechanisms. First, the inhaled irritant directly damages epithelial cells. Many components of cigarette smoke are toxic – carbon monoxide, benzene, cyanide, etc. – and could turn on apoptotic pathways. A second possibility is irritants are sensed by lung resident leukocytes – mainly macrophages and mast cells – and epithelial cells, which release factors that recruit polymorphonuclear cells from the blood. These

resident and recruited cells are then triggered to produce effector molecules that exact damage on the lung epithelium.

Evidence suggesting that Toll-like receptors (TLRs) may be involved in sensing these noninfectious irritants is mixed. A study published in 2005 found that TLR 2 and 4 were involved in the inflammatory response to sterile stimuli, specifically monosodium urate monohydrate (MSU) crystals – responsible for gout – and subcutaneous air injections (10). Bone marrow-derived macrophages of TLR2 knockout (KO) mice and TLR4 KO mice were found to produce significantly less IL-1 β and TNF- α (10). Another study implicated TLR 3 in the inflammatory response to ischemia under sterile conditions. 24 hours after the ischemic event, TLR3 KO mice had TNF- α levels approximately one-third those of normal mice (11). This is particularly interesting in light of evidence for hypoxia following tobacco smoke inhalation (12). TLRs classically sense microbial molecular patterns. The idea that TLRs might have sterile triggers as well is provocative. Curiously, similar studies found no evidence that TLRs were stimulated by sterile injury (13).

The family of nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are intracellular receptors, unlike TLRs which are found at the cell surface and in endosomal membranes. NLRs typically cooperate with TLRs to generate appropriately focused inflammatory and apoptotic responses. The literature generally supports a role for NLRs in sterile inflammation. Dostert and colleagues published in 2008 that inhaled asbestos and silica activate the innate immune system via Nalp3, a member of the NLR family (14). Asbestos and silica inhalation are documented risk factors for emphysema.

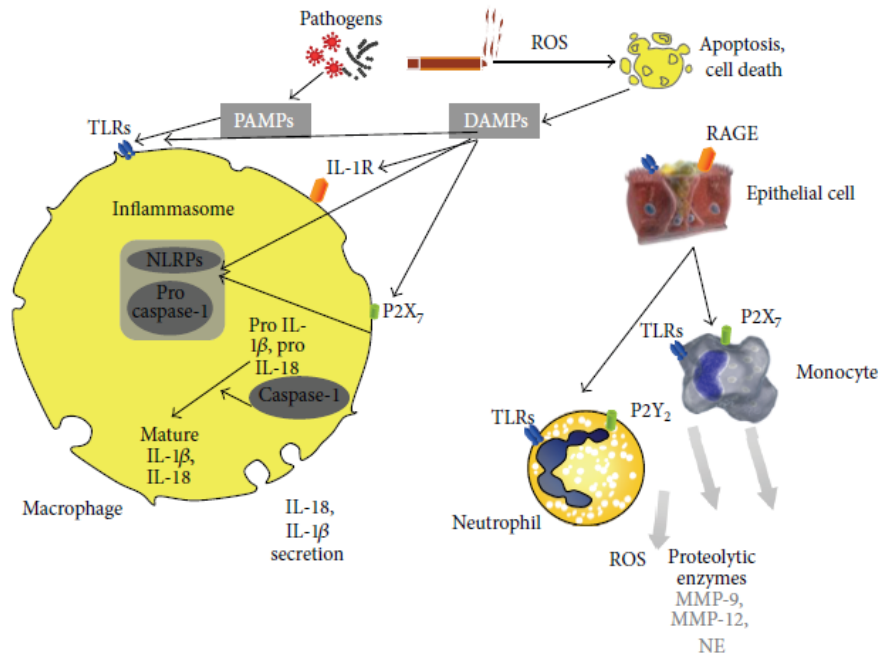
While asbestos and silica are particulate and thus diverge from the suspected stimuli in tobacco smoke, they do induce DAMPs like reactive oxygen species (ROS's) which are known triggers of Nalp3. Once activated, Nalp3 inflammasomes can stimulate dramatic increases in IL-1beta and other chemotactic factors. In Dostert's research, it was found that Nalp3 KO mice had a diminished cellular response to the irritants, including significantly fewer neutrophils (14). A more recent study took this idea one step further. Nalp3 KO mice were found to have better outcomes to acute hypoxic injury as measured by increased epithelial cell survival (15).

Other receptors involved in sensing signs of danger have been examined as well. The IL-1 receptor and its accompanying adaptor protein, MyD88, have been linked to sterile injury as drivers of inflammatory responses. Gouty inflammation in response to monosodium urate (MSU) crystals is dependent on MyD88 – animals deficient in MyD88 do not develop the pathology (16). Similarly, IL-1R KO mice also have severely stunted inflammatory responses to MSU (16). Administering antibody to block IL-1 interactions with its receptor has the same effect (16). While MSU is a good model for studying sterile inflammation, it is unclear how applicable this model is to the sterile inflammation suspected of driving emphysema. Another trigger, dying cells, might be a more logical connection. Smoking tobacco does result in lung cell death (17). A 2007 study of dying cells confirmed that IP injections produced marked neutrophilic inflammatory responses in mice (18). The study then demonstrated that IL-1R KO mice and MyD88 KO mice did not develop the expected inflammation. It is interesting that the IL-1 pathway is a requirement of neutrophilic inflammation only in response to

sterile stimuli; it is not required when infection is driving the response. While interesting, a single IP injection of dying cells is not equivalent to smoking a cigarette, for example, and any extrapolation of these results to emphysema would be premature.

Another key point to keep in mind is that much of the research cited above is done on acute models. Many diseases of sterile inflammation are in fact chronic – emphysema being a prime example. The current body of literature should be used to guide the discovery of better chronic disease models.

Figure 2: Lung inflammation due to cigarette smoke involves inflammasome activation.



To summarize, *how* cells lining the lumen of the lung are damaged in response to irritants like tobacco smoke is not clear. Apoptosis could be triggered directly by irritants and free radicals, or irritants could be detected and spur on inflammatory pathways known to cause cell damage. Evidence for TLRs, IL-1R, and MyD88 as cellular

sensors for detecting irritants was presented; how they may fit into the greater schema is illustrated in Figure 2 (19).

DAMPs and Tissue Damage

Cell death and the initiation of inflammation represent only the beginning of a long and complex process, a process that can culminate with the development of emphysema. The next step in this process is propagation of inflammation. Cells, their cytokines and effector functions will be discussed next.

Tissue damage drives the discharge of damage-associated molecular pattern molecules (DAMPs) into the extracellular compartment. These include pro-IL-1 α , heat shock proteins, hyaluronan fragments, ATP, adenosine, DNA, uric acid, and heparin sulfate – generally molecules that are not exposed to the extracellular compartment under normal conditions either because they are retained within cells or have lost their recognizable structure. Recent work investigating high-mobility group box 1 (HMGB1), an intracellular molecule with DNA-binding sites and anti-apoptotic activity, has unmasked its huge immunogenic potential if released into the extracellular milieu, as occurs in sterile injury (20). HMGB1 interacts with TLR2, RAGE, and TLR4 to elicit the expression/release of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM-1), TNF- α , IL-8, and monocyte chemotactic protein 1 (MCP-1), among other factors (20), which together activate endothelial cells and recruit and activate immune cells. However, HMGB1 is not released any time a cell dies. Like other intracellular molecules that are highly immunogenic if they escape into the extracellular

compartment (CpG DNA and RNA are other examples), HMGB1 is only released during necrotic or damage-induced cell death. During apoptotic death, HMGB1 is sequestered out of the reach of PRR detection. An in vitro study used cigarette smoke to show both apoptotic and necrotic cell death occur post-exposure, and these results have been confirmed in subsequent studies (17). HMGB1 is a DAMP widely recognized for its involvement in many disease processes from cancer to COPD. Damaged cells also have extensive inflammasome activation (the Nalp3 inflammasome was mentioned above). Inflammasomes recruit cytosolic caspases, one result of which is the cleavage and release of IL-1 β .

Both IL-1 β and DAMPs – the primary source being lung epithelial cells that bear the brunt of exposure – trigger a number of inflammatory pathways. Resident leukocytes sense DAMPs via TLRs, NLRs, C-type lectin receptors, and RIG-I-like receptors (21). Receptor engagement leads to signal transduction and amplification. Pre-made effector molecules can be mobilized, various receptors confined to endosomes are circulated to the surface, cell motility is altered, and transcription factors – namely NF- κ B – turn on inflammatory genes whose products dictate the nature of the response. The initial response from tissue macrophages is comprised of TNF- α , IL-1, and IL-6. These cytokines diffuse through the interstitium to the local vasculature where they can begin recruiting other leukocytes. In addition to calling in blood monocytes and granulocytes, these cytokines participate in feedback loops that amplify the local inflammatory response and induce IL-8 production. IL-8 is a potent chemoattractant for neutrophils. Research suggests that TNF- α is responsible for 70% of cigarette smoke-

induced emphysema in a chronic exposure mouse model (22). This is compelling evidence for a Th1 effector profile driving the disease process. But TNF- α is merely a tool used to communicate between effector cells. It is the effector cells themselves that are capable of propagating inflammation and damage.

Effector Cells

The existing body of literature on emphysema focuses on four main effector cell types – epithelial cells, neutrophils, monocytes/macrophages, and lymphocytes – and reports varying contributions, some well-established, others more controversial, of each cell type to the initiation and propagation of emphysema. The evidence for each cell type will be discussed in turn in the following section. Note that while eosinophils do play a significant role in asthma and allergy there is little evidence for their role in emphysema. Eosinophilia in COPD airways is not uncommonly reported. Clinically, COPD does not respond well to corticosteroids whereas patients with “eosinophilic COPD” who receive treatment do report improvement (23). However, concomitant asthma is frequently used to explain this phenomenon. Eosinophils will not be discussed further.

Neutrophils

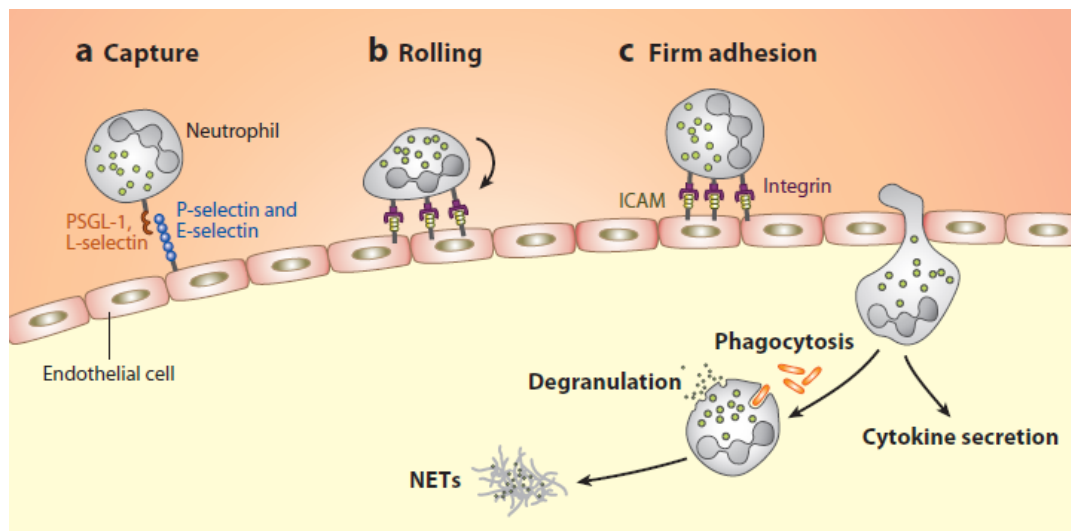
Neutrophils or polymorphonuclear cells (PMNs) are the first responders to both sterile and infectious damage. In the case of cigarette smoking, neutrophils migrate into the lungs in response to toxin-induced damage and oxidative stress. Neutrophils are

terminally differentiated phagocytic granulocytes originating from bone marrow pluripotent hematopoietic cells that circulate in the blood for 6-8 hours before unobtrusively apoptosing (24). However, in vitro experiments have shown that neutrophils can survive much longer – and retain their functionality – in the presence of IL-1 β or LPS (25). Despite being short-lived in nature, neutrophils are abundant, comprising 70% of the blood leukocyte population in adult humans and 30% in mice (24). Their high numbers are maintained by the bone marrow pool and turnover is rapid to offset apoptotic loss. The characteristic neutrophil granules contain antimicrobial peptides that are made continuously and contained within fused vesicles. Degranulation – the primary effector function of neutrophils – is preceded by many sequential activation events. These events rely on TNF- α , IL-1, and IL-8, among other PAMPs and DAMPs, which are only present in the context of injury or infection. Neutrophil granules are kept strictly segregated from the body in the absence of these signals.

The aforementioned cytokines are recognized by endothelial cells lining pulmonary vessels and trigger the display of adhesion molecules for recognition by PMNs. PMNs circulate the blood at a high velocity. Like other leukocytes, priming is an important step in activation and can alter the potency of the effector response. Neutrophils are primed by cytokines such as TNF- α and by hypoxia (2). Selectin and integrin recognition slows these cells down as they weakly bind to blood vessel walls. In the presence of the proper signals, weak interactions are exchanged for stronger ones. PMNs can then migrate across vessels into tissues down cytokine concentration

gradients to the origin of inflammation (reviewed in (24)). Endothelial cells stimulated with TNF- α and IL-1 produce granulocyte-macrophage colony stimulating factor (GM-CSF, CSF2), a cytokine that is known to activate neutrophils and potentiate the production of reactive oxygen species (26). Neutralizing GM-CSF with antibody stunts ROS production in neutrophils by 50% to 70% (26). This suggests that *how* neutrophils are recruited can have an impact on downstream effector function. IL-8 (CXCL8) is a powerful chemoattractant that directs PMNs from the vasculature into the tissue (27). After CXCR1 or CXCR2 – receptors for IL-8 – are engaged and extravasation is complete, leukotriene B4 (LTB4), the complement fragment C5a, and DAMPs such as mitochondria-derived formylated peptides serve as end-stage chemoattractants (25). The recruitment and effector functions of neutrophils are illustrated in Figure 3 (24).

Figure 3: Neutrophil recruitment, extravasation, and effector functions.



The PMN response to injury is rapid. For example, after a mouse aspirates elastase, thousands of neutrophils are drawn into the lung in mere hours – their

numbers peak about 24 hours after the initial stimulus. Depending on the intensity of the inciting stimulus neutrophil counts can reach into the hundreds of thousands.

Neutrophils have a potent arsenal of effector molecules including reactive oxygen species (ROS's), proteases, defensins, and antimicrobial peptides that are very effective against a spectrum of pathogens. The neutrophil is a key component in the innate immune response against bacterial (28), viral (29), fungal (28), and parasitic (30) pathogens. Neutrophil-deficient animals suffer from recurrent bacterial infections (31). While it is well-documented that neutrophils play an important role in the early stages of wound repair through the release of pro-resolving lipid mediators such as lipoxin A4(LXA4) and resolvins (25), the beneficial and detrimental roles that neutrophils play during chronic inflammation is less clear. Because innate effector functions are by definition nonspecific, collateral damage to the body occurs. During acute infection this collateral damage is a small price to pay. During a chronic infection or sterile inflammation, this remittent damage becomes a significant source of the pathology and it often becomes difficult to tease apart which is the main offender. Illustrative of this conundrum is the involvement of oxidative stress in tissue damage (20). ROS's directly damage tissue, provoking the release of DAMPs that in turn induce inflammatory cascades (20). Neutrophils and other leukocytes can be the source of ROS's, but more relevant to the discussion of emphysema, ROS's can come directly from cigarette smoke (32). Not only do smokers have a higher ROS load, they also have fewer antioxidants to relieve the tremendous oxidative stress (32). Given this, it is difficult to prove that neutrophils release these ROS's and proteases upon migration to the lung. Neutrophils

are not the only cells to release these effector molecules; and ROS's are degraded by antioxidants and proteases quickly inactivated by serum antiproteases soon after release. There is to date no confirmed mechanism explaining why neutrophil elastase KO mice show 59% protection from emphysema (33).

A newly characterized effector mechanism of neutrophils is the release of neutrophil extracellular traps (NETs), or the extracellular fibril matrices formed when neutrophils release their DNA and proteins. Immunofluorescent imaging shows NETs are present four hours after LPS administration (21). Following activation, neutrophils can release NETs in as little as ten minutes (34). NETs work by trapping infectious agents, scavenging debris, and concentrating proteases and anti-microbial peptides to efficiently eliminate these threats with minimal damage to the host. In a mouse model of sepsis, blocking NET formation reduced *Escherichia coli* clearance from the blood and resulted in greater dissemination (21). While beneficial in the context of infection, NETs are negatively implicated in sterile damage. Release of NETs is associated with IL-8 and, more recently, with HMGB1 through interactions involving TLR4 (35). Recalling that HMGB1 is associated with necrotic death, which is in turn associated with smoking, NETs could very well play a role in emphysema. Many components of NETs, such as extracellular histones, are antigenic and require swift degradation and clearance. Serum DNases play some role in NET degradation, but twelve hours incubation with plasma from healthy donors is not sufficient to fully clear NETs (36). Complement factor C1q enhances NET degradation by DNase (36). Macrophages are also involved in NET

clearance, with eighteen hours incubation with monocyte-derived macrophages cutting NET activity in half (36). The release of NETs may be rapid, but clearance is slow.

It is also noteworthy to mention other chronic lung diseases exhibiting neutrophilia comparable to or more extensive than that seen in emphysema, such as cystic fibrosis, do not involve alveolar destruction. Other factors must be involved. A 2012 review of neutrophils in COPD presents evidence that hypoxia – induced by smoking – alters neutrophil survival and function, delaying apoptosis and enhancing the release of proteases (2). The idea that cigarette smoke might durably alter the phenotype of immune cells is key to understanding why lung function continues to decline even after smoking cessation (2). It might also explain why patients with COPD struggle to clear bacterial lung infections despite their neutrophilia. The detrimental effects of cigarette smoke are shared by lung macrophages. A 2009 study published that cigarette smoke impaired the ability of macrophages to phagocytize apoptotic neutrophils (37). This dysfunctional phagocytosis could be linked to an excess of neutrophil elastase, which has been shown to cleave certain complement fragments and receptors involved in phagocytosis (2). In vivo mouse studies have shown apoptotic cells that are not promptly removed from the inflammatory environment are subject to secondary necrosis releasing additional DAMPs into the lung (38).

Hoenderdos and Condliffe proposed in their 2013 review that the magnitude and dynamics of neutrophil transepithelial flux influence epithelial integrity (2). This migration from pulmonary capillaries to the lung lumen promotes both neutrophil elastase-mediated epithelial cell apoptosis and β -catenin-mediated membrane repair

signals (39)(40). Following the biological paradigm that balance among interdependent pathways is critical to maintain homeostasis and health and where imbalances exist disease soon follows, perhaps the influence of cigarette smoke on leukocyte function, apoptosis, and phagocytosis disrupts this delicate balance and leads to a chronic inflammatory response.

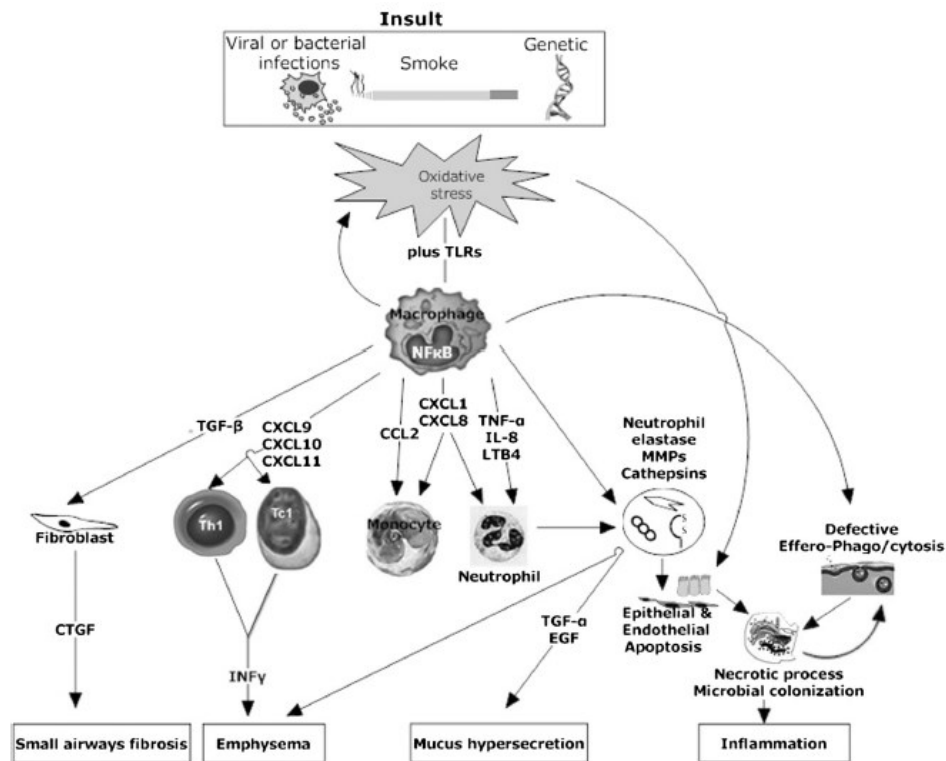
Monocytes and Macrophages

Monocytes and macrophages are derived from hematopoietic stem cells in the bone marrow, and the classic paradigm is of a monocyte circulating in the blood until being called into the tissues at which point the monocyte differentiates into a tissue macrophage. These macrophages work alongside tissue resident dendritic cells and mast cells as sentinels, surveying the tissues and responding to damage of both sterile and infectious origin. In emphysema, these macrophages are the first to release TNF- α , IL-1, IL-6, and IL-12 that initiate the complex cascade of inflammatory molecules – a crucial step in the pathologic process.

Recent evidence contradicts that blood monocytes are the source of tissue resident macrophages. In the evolving paradigm shift, macrophages are now believed to seed the tissues early in embryonic development, maintain themselves through local proliferation, and that the parent macrophage's differentiated state is passed on to daughter cells (41). Such inheritance of an effector phenotype has strong implications for disease – the inflammatory environment of a past illness could influence the course of future disease in that same organ. In homeostasis, depleting circulating monocytes

has no significant effect on tissue macrophage populations (42). Many lineage-tracing models found that peritoneal, alveolar, splenic, skin, mucosal and liver macrophage populations did not have markers of monocytic origin (41). Of more relevance to the lung, 1-10% of alveolar macrophages are proliferating under steady-state conditions, and a “strong expansion” of alveolar macrophages was reported in response to diphtheria toxin and clodronate-depletion (42). Considering GM-CSF, a cytokine growth factor produced by lung epithelial cells in response to cigarette smoke-induced damage, it would not be surprising if lung macrophage were found to proliferate in emphysema as well. Most of the research investigating factors that maintain tissue macrophage populations has been done in disease-free models. The extent of monocyte recruitment in disease states such as emphysema is unknown.

Figure 4: Macrophages are a central regulator of the immune response to cigarette smoke.



Macrophages release mediators that coordinate granulocyte (particularly neutrophil), dendritic cell, epithelial cell, and lymphocyte responses. The cytokine profile in emphysema – namely IFN- γ , TNF- α , IL-1, IL-6, IL-12 – suggests that lung macrophages are classically activate (M1), a phenotype associated with NF- κ B activation, enhanced phagocytic activity and ROS production (43). Macrophages from smokers and COPD patients show increased proliferation and prolonged survival, mechanisms which might account for the increase in CD68+ macrophages in the bronchial submucosa of COPD patients (44). In culture, alveolar macrophages harvested from smokers release more MMP1 (a broad-spectrum collagenase) and MMP9 suggesting their inflammatory potential (44). While neutrophil elastase KO mice showed 59% protection from emphysema (33), macrophage elastase KO mice showed a more impressive 100% protection from cigarette smoke-induced emphysema (45). Such complete protection in the absence of macrophage elastase underscores the importance of these cells in initiating emphysema. Figure 4 depicts the many reported roles that macrophages play as a central regulator of the immune response to cigarette smoke (43).

Unlike neutrophils, macrophages localize to the site of alveolar destruction (4). They are present in the lung parenchyma, sputum, BAL fluid, and airways of COPD patients (4). Most notably, a 2001 study compared lung resection samples from patients with similar smoking histories, but different degrees of emphysema, and found that parenchymal macrophage levels were elevated 25-fold in smokers with emphysema compared to smokers without emphysema (8). This is a striking

observation, but the possibility that macrophages are responding to damage and not necessarily causing it cannot be eliminated. Additionally, the extent of emphysema in resected lungs was observed to correlate with macrophage levels, but not with neutrophil levels (1).

IL-18 is an IFN-gamma-inducing, proinflammatory cytokine produced by macrophages and associated with a Th1 phenotype. Besides being up-regulated in COPD patients compared to healthy smokers and nonsmokers, studies in mice have demonstrated IL-18 is important in the disease process (19). IL-18R KO mice show decreased inflammation and a 51.5% reduction in emphysema as measured by alveolar chord length (46). IL-18 in conjunction with IL-12 activates CD8+ T cells and correspondingly cell-mediated immunity. Elevated CD8+ T cell counts are commonly reported in emphysematous lungs, more so than any other lymphocyte population, and in fact continue to rise as the disease progresses (19). The repercussions of this will be considered when autoimmunity as a potentiator of emphysema is discussed.

A possible connection between dysfunctional macrophages and chronic inflammation is the previously discussed NET, or neutrophil extracellular trap. A recent publication claims that physiological amounts of DNase I – a key enzyme in the cleanup of chromatin released from necrotic cells – are not sufficient to fully degrade NETs (36). Macrophages are proposed to engulf and silently clear NETs from the extracellular environment; however, recall that cigarette smoke impairs phagocytosis in macrophages potentially allowing NETs to persist in an environment where antigen surveillance, presentation and recognition are ongoing. The proteins and DNA that

make up NETs are normally intracellular and if recognized by the adaptive immune system could result in autoimmune activation and disease. In fact, a subset of patients with systemic lupus erythematosus (SLE) – regarded as the prototype autoimmune disease – have impaired degradation of NETs and anti-nuclear autoantibodies (25). Autoantibodies to dsDNA or histones are features of many autoimmune diseases, and if evidence for autoimmunity in emphysema accumulates this might become of greater interest.

Lymphocytes

In the past decade or two, a new mechanism for emphysema was proposed that argues adaptive immunity and not an innate protease imbalance is the primary driver of emphysema. Beginning with The Lung Health Study – a first-of-its-kind, longitudinal study following 5,000+ smokers over eleven years – the observation was made that between 8-10% of former smokers were still experiencing a decline in lung function (3). Removing the insulting stimulus did not stabilize their condition. This was suspiciously similar to the behavior of autoimmune diseases where immunologic memory and ever-present self-antigens lead to a chronic, difficult to treat condition. Around the same time when The Lung Health Study data was being compiled, another lab published that CD8+ T cells had an elevated presence in smoker lungs (3). These two findings sparked a flurry of research, which has affected a dramatic shift in our understanding of emphysema (3).

The evidence for autoreactive antibodies – i.e. anti-epithelial and anti-elastin – in emphysema patients is scarce, and what exists is often contradictory, but conversely studying autoreactive antibodies is inherently more difficult than studying autoreactive T cells (3). The extent of B cell infiltration in the airways does correlate with the severity of COPD, as demonstrated in resected lung tissue from patients classified by Global Initiative for Chronic Obstructive Lung Disease (GOLD) standards (47). Additionally, the progression to severe and very severe COPD is associated with an increase in airway lymphoid follicles, which are structurally important for B cell activation, selection, and antibody production (43). However, exacerbations triggered by opportunistic infection as the integrity of the pulmonary epithelial barrier fails are also associated with severe COPD. Infection and not autoimmunity could be driving the organization of lymphoid follicles in the lung. Any verdict on B cell involvement would be premature, so T lymphocytes and cell-mediated immunity will be the primary focus of this section.

When emphysema does not resolve or stabilize in former smokers, what continues to trigger the release of MMPs and elastase? IFN-gamma indirectly induces the elastin-degrading MMP12 (48). IL-17A indirectly induces neutrophil and dendritic cell chemoattractants and MMP12 (49). IFN-gamma is the signature cytokine of the Th1 subset of CD4+ T cells, and IL-17A is characteristic of Th17 cells. No Th2 markers are evident. T-regulatory cell counts are also markedly low, translating to low levels of IL-10 and only limited immunomodulatory mechanisms to counter the extensive inflammation (19). Indeed, T cells isolated from emphysematous lungs are largely Th1 (4), and lung myeloid dendritic cells isolated from emphysematous lungs have been

shown to induce IFN-gamma and IL-17A secretion from CD4+ T cells in vitro (49). There is also a positive correlation between the degree of alveolar destruction, the severity of clinical symptoms, and the number of T cells (4). Importantly, the combination of Th1 and Th17 cells has been experimentally linked to many autoimmune diseases in humans (3).

This body of evidence is only meaningful if T cells are present and activated in emphysematous lungs – no adaptive effector functions can take place without recognition of a specific antigen, and in the case of the autoimmunity hypothesis, a specific self-antigen. Early studies have demonstrated that smokers have increased numbers of dendritic cells in airway and alveolar walls, providing a mechanism for antigen presentation and T cell activation (4). Activated CD4+ T cells have themselves been quantified and found elevated in the airway walls of COPD patients (44). A 2007 publication presented evidence that peripheral blood CD4+ T cells isolated from emphysema patients produced significant levels of IFN-gamma and IL-10 in response to treatment with elastin, but not collagen or albumin; a response that was attenuated when MHC II molecules were blocked (50). Control subjects did not respond to elastin, collagen, or albumin. Furthermore, the IFN-gamma response was found to positively correlate with the severity of emphysema. Plasma samples from study volunteers were also screened for autoreactive antibodies and anti-elastin antibodies were found to be significantly elevated in emphysema patients but not controls. Again, this report of an autoreactive humoral response should be considered alongside similar studies that did not detect a significant difference between patients and controls. The opinion

presented in a 2012 review by Kheradmand and colleagues was that differences in antibody testing rigor and inclusion criteria and stratification of study participants account for these differences (3). In a similar demonstration of the destructive potential of autoreactive CD4+ T cells, mice who received adoptive transfers of pathogenic CD4+ cells developed emphysema in the absence of any other stimuli (51). The same study showed that mice inoculated intraperitoneally (IP) with xenogeneic epithelial cells also develop emphysema (51).

CD8+ T cells exact direct damage on cells when activated through the release of cytotoxic mediators such as perforin and granzymes. CD8+ T cell activity is evident in sputum and BAL fluid of smokers – both perforin and granzyme B are elevated (44). These enzymes trigger caspase-mediated apoptosis, and indeed there is a positive association between the number of CD8+ cells and the number of apoptotic cells in alveolar walls (44). CD8+ T cells isolated from the sputum of COPD patients demonstrate increased perforin expression and cytotoxic activity (44). In 2007, a study published that CD8 KO mice smoked twice daily for six months did not develop emphysema and showed only limited inflammation compared to their wild-type (WT) and CD4 KO comparators (52). Both WT and CD4 KO mice developed emphysema. This phenomenon was attributed to IFN-gamma-inducible protein-10, a CD8+ T cell product that induces MMP12. It is suspicious that CD4 KO mice still developed emphysema because if blocking the CD8 response is protective as the article suggests, and knowing that CD4+ T cells prime CD8 effector function, it would be expected that CD4 KO's would

also show some protection from emphysema. Regardless, the authors concluded that CD8 T cells were required for alveolar destruction in cigarette-smoked mice.

The literature does not agree on a conclusive role for lymphocytes in emphysema, clearly illustrated by experiments with RAG1 KO mice that lack both T and B cells – these mice still develop smoke-induced emphysema after six months of exposure (53). The RAG1 KO experiments were done in BALB/c mice; the CD8+ KO experiments used C57BL/6 mice – a confounding variable that could explain the outcomes to a common research question. The reality of models is that they are imperfect. Lymphocytes may not be necessary for the development of emphysema in either humans or mice, or this could be a point at which the pathogenesis in the model diverges from the pathogenesis in humans. On that note, alveolar damage in smoked mice does resolve after smoking cessation, whereas in humans this is clearly not the case, putting the validity of the cigarette smoke model in question (3).

Epithelial Cells

Airway epithelial cells experience the brunt of the damage from cigarette smoke and other inhaled environmental irritants. These cells are effective mediators of the barrier function between the body and the outside world – they secrete mucus, antimicrobial peptides, antioxidants, antiproteases, and transport IgA into the lung lumen (4). However when damaged, epithelial cells release DAMPs and alarmins into the lung parenchyma that are recognized by resident macrophages and innate lymphoid cells. In response to cigarette smoke and the resulting oxidative stress epithelial cells

secrete inflammatory mediators such as TNF- α , IL-1 β , GM-CSF, and IL-8 (4). While epithelial cells are not classically considered immune cells, their role in the inflammatory process cannot be overlooked. Epithelial cells account for 16% of all cells in the lung, which is more than double the maximum estimates for alveolar macrophages (54).

An upregulation of pro-apoptotic and various antioxidant genes is seen in smoker lung epithelium (44). Epithelial cells also upregulate proteins that fight ROS damage – namely unfolded protein response (UPR) proteins – in association with chronic smoking (44). From a chronic care perspective, growth factors are a particularly troubling aspect of the epithelial response to cigarette smoke. An irreversible activation of the epidermal growth factor receptor (EGFR) cascade occurs in smokers, and overexpression EGFR is associated with the development of lung cancer (44).

Overexpression, predominantly in epithelial cells, of the receptor for advanced glycation end products (RAGE) was observed in smoker lungs with emphysema but not in healthy smokers and nonsmokers (55). RAGE interacts with extracellular HMGB1 – a highly immunogenic intracellular protein released from damaged and necrotic cells that was previously discussed. Overexpression of RAGE was associated clinically with a decline in lung function, and on a molecular level with an increase in oxidants (NO), a decrease in antioxidants (glutathione), and an increase in NF- κ B-dependent proinflammatory cytokines (55). The lab then confirmed these results could be reversed with pretreatment with anti-RAGE antibody. It is presently unknown whether RAGE expression is primarily dictated by genetic or environmental factors.

From the above discussion on effector cells it is easy to see how epithelial damage can lead to inflammation, which in turn can lead to more damage and more inflammation, and so the vicious chronic cycle is established. This is where some immunologists propose the adaptive immune system comes in, escalating a simple condition of chronic inflammation to one that extinguishes any hope of resolution – autoimmunity. As previously discussed, persistent tissue damage allows intracellular or modified extracellular fragments to accumulate and mingle with antigen-presenting cells (APCs) and lymphocytes. These fragments are not recognized by the adaptive immune system – it has not been tolerized to them – and thus autoimmunity can result. There is substantial evidence suggesting emphysema is *instigated* by innate immune mechanisms but that the adaptive mechanisms – cytotoxicity and antibody – help perpetuate the disease. Even so, if innate mechanisms start the disease process they are a possible point of intervention and deserving of further investigation.

The Elastase Model of Emphysema

The complex etiology of emphysema and COPD mandates the use of in vivo animal models; rodents, guinea pigs, dogs, and monkeys, to name a few. Mice are by far the animal of choice and not surprisingly so considering their biology is well defined, they can be genetically engineered, a wide array of antibody reagents are available, and they are relatively affordable.

In mice, emphysema can be induced with particulates (cigarette-smoke extract, silica), proteases (elastase), oxidants, cigarette smoke, and certain genetic mutations.

Cigarette smoke might seem the intuitive choice given that smoking is the primary cause of emphysema in people. However, mice exposed to cigarette smoke take many months before emphysematous changes become manifest, a costly limitation, and the emphysema is mild even when the exposure is high (56). The cigarette smoke model also does nothing to tease apart the epithelial damage-driven, immune-mediated pathology from the direct effects of cigarette smoke-induced oxidative stress. In contrast, emphysema in the elastase model is dose-dependent ranging from mild to severe depending on the number of enzymatic units administered. An inexpensive, single dose of elastase is enough to cause emphysema in mere weeks. The direct oxidative effects of cigarette smoke have no influence in the elastase model, allowing emphysema as a disease of immune dysregulation to be studied with fewer confounders. But the elastase model comes with its caveats. Some evidence suggests elastase-induced emphysema diverges mechanistically from cigarette smoke-induced emphysema, which makes for a convoluted extrapolation from mice to people. While symptoms of systemic disease are evident in clinical emphysema as well as both the elastase and cigarette smoke murine models, emphysema *progresses* in people over time even after smoking cessation. Quite the opposite of progression, lesion *resolution* is seen in the cigarette smoke model. Elastase-induced emphysema shows lesion maintenance with evidence of progression being questionable (56). Moreover, is it valid to study a disease of chronic environmental exposure using a single-insult, acute model? Emphysema is not the only chronic disease facing this research discrepancy. In fact, as

today's sphere of public health becomes predominated by chronic diseases this is fast becoming the ultimate question of modern medical research.

The parameters of study for emphysema are alveolar destruction and reduction in ventilation and gas exchange; they have been defined from the clinical criteria.

Historically, mean airspace chord length (L_m) has been the measurement of choice for alveolar destruction. Mean airspace lengths, or the average distance between luminal walls, are obtained by random sampling of H&E stained lung histology sections.

Emphysema is marked by large chord lengths because the alveolar septa dividing up the airspace and increasing the surface area for gas exchange are greatly reduced in number. However, this approach is not perfect and has received criticism from experts in the field. The main criticisms are, 1) L_m cannot distinguish between tissue distension – from over-inflation or loss of elasticity – and tissues destruction, 2) random sampling does not take into account the anisotropy of emphysema, and 3) L_m does not always predict lung function deficits. A 2008 article by W. Mitzner argues that L_m is still useful and taking into account lung volume, sampling 10,000 or more chord lengths, and measuring L_m in conjunction with lung function allows researchers to avoid these pitfalls (57).

Reductions in ventilation and gas exchange can be quantified by a variety of tests for lung function. Diffusion coefficients are a standard measure of gas exchange capacity, which would be impaired anytime the surface area for gas exchange was compromised. For such tests, a highly soluble gas (carbon monoxide) and insoluble gas (neon) are inhaled at known concentrations and expired air is analyzed for changes in

concentrations. Additionally, forced expiratory volume (FEV1), total lung capacity (TLC), functional residual capacity (FRC), and residual volume (RV) are all clinically accepted and useful in grading COPD severity. These and other tests of lung function are reviewed in “Outcome measures in chronic obstructive pulmonary disease (COPD): strengths and limitations” by Glaab, Vogelmeier, and Buhl (58).

Hypothesis and Justification

The purpose of this research is to directly assess the role – be it protective, detrimental, or neutral – that neutrophils play in the development of IT elastase-induced emphysema. Lung histology and function will be the primary methods of emphysema assessment in our 21-day, elastase- BALB/c mouse model, and neutrophil involvement will be determined by comparing these parameters in neutrophil-intact and neutrophil-depleted mice. Because neutrophils are characteristically the first responders to damage or infection we intend to follow the acute BAL cellular response to IT elastase and more precisely define the window of neutrophil activity in the lung. The major effector cell types – neutrophils, macrophages, and lymphocytes – will be followed over various early time points in the BAL fluid of elastase-treated mice. Using this data we will target our antibody depletion of neutrophils during their frame of influx and track changes in other cell types associated with depletion.

We hypothesize that neutrophil depletion will worsen the elastase-induced emphysema pathophysiology – that neutrophils will be found to be protective. We choose this stance for many reasons.

Neutrophils are important in tissue remodeling and repair, and they have in their arsenal of effector mechanisms the newly discovered NET which has not been characterized in models of sterile injury. Some studies have shown PMNs to be beneficial in colitis (reviewed in (59)), a chronic inflammatory disease of the GI tract, but such evidence is limited in the context of the lung.

Glucocorticoids inhibit neutrophil adhesion to vascular endothelium and limit transmigration into the parenchyma (21). It has been mentioned that glucocorticoids are ineffective in treating COPD. This suggests that neutrophil recruitment is not significantly involved in the maintenance of disease in emphysema.

A 2013 review by Kolaczowska and Kubes presented the idea of separate but inducible inflammatory and anti-inflammatory neutrophil states (21). Lipoxin A4 (LXA4) is a steroid associated with the resolution of inflammation and it has been shown to act on neutrophils, inhibiting neutrophil migration and recruiting macrophages to clear apoptotic neutrophils (21). In COPD patients, serum amyloid A (SAA), an inflammatory lipoprotein opposing the action of LXA4, is elevated and LXA4 is disproportionately low compared to healthy controls (60). Furthermore, the same lab showed that SAA correlates with IL-8, neutrophil recruitment and granule release (60). Perhaps low LXA4 prevents neutrophils from adopting an anti-inflammatory phenotype in the context of emphysema.

Arginase I is a component of neutrophilic granules that has been found to inhibit T cell activation (21). It has been proposed that autoimmunity continues to drive emphysematous changes even after smoking cessation (3). Depleting neutrophils could

have an impact on the magnitude of the T cell response and thus increase the risk of developing autoimmunity, especially in habitual smokers whose lungs are chronically producing DAMPs in response to noxious stimuli. Conversely, neutrophil-derived chemokines CCL2, CXCL9 and CXCL10 help recruit TH1 and TH17 cells to sites of inflammation (25).

A 1999 article published that neutrophil antibody depletion did not protect rats from developing emphysema in a cigarette-smoke model, but mean airspace chord lengths and immunologic assays for elastin fragments in lavage fluid found monocyte/macrophage depletion was protective (61). In contrast, a 2003 study showed that neutrophil elastase KO mice were 60% protected from cigarette smoke-induced emphysema (33). The length of smoke exposure was different (two versus six months), the method of depletion different (once-weekly administration of antibody targeting neutrophils versus genetic knockout of neutrophil effector function), and the animal model was different (rat versus mouse). Given two very different study designs, it is hardly surprising the results do not agree; but perhaps a discovery is hiding in their differences. Neutrophil elastase KO mice had fewer recruited neutrophils, fewer recruited monocytes, less macrophage elastase, and smaller airspaces. In both experiments reduced recruitment of monocytes was associated with protection. Summarizing these two studies we find that removing neutrophils *and* neutrophil elastase does not protect against emphysema, but removing only the elastase does protect. Perhaps too much emphasis is placed on neutrophil elastase and too little on other neutrophil products.

While the role of neutrophils in emphysema has been previously tested, no work has been done in the elastase model and varying reports exist for the cigarette smoke model. Beyond expanding our understanding of the elastase model, the original research presented in the subsequent sections will shed additional light on an unsettled question: How do neutrophils contribute to emphysema?

MATERIALS AND METHODS

Mice

BALB/c and C57BL/6 male mice, 6-8 weeks of age, were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were bred and housed within barrier filter-top cages, provided food and water ad libitum, and exposed to a 12-hour light/dark cycle. All animal procedures performed in this study were approved by the Johns Hopkins Animal Care and Use Committee (Baltimore, MD, USA), and were in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals federal guidelines. Within experiments mice were age-matched; between experiments slightly different ages were used depending on availability but the range was limited to 7-12 weeks.

Elastase Administration

Mice were challenged via intra-tracheal (IT) aspiration with three enzymatic units (U) of porcine pancreatic elastase (PPE). Mice were first anesthetized with a mixture of 1.75 mL isoflurane and 3.25 mL propylene glycol. A piece of cotton gauze was soaked with the 5 mL isoflurane mixture and placed at the bottom of a bell jar. A mouse was then enclosed in the bell jar and monitored until its breathing rate had slowed to approximately one breath per second and this rate was maintained for about ten breaths. At this point, the mouse was considered properly anesthetized, removed from the chamber and placed on its back at a forty-five degree incline. Forceps were used to gently extend and stabilize the tongue. Using a pipet, 50 μ L of phosphate buffered

saline (PBS) containing 3 U of PPE was placed at the back of the throat. A gloved finger was used to cover the mouse's nose, forcing the mouse to inhale through the mouth and aspirate the PPE into the lungs. Successful aspiration was presumed when the mouse had taken a few breaths – evidenced by expansion of the chest cavity – and the oropharynx appeared void of PPE solution.

PPE-treated mice were returned to their cages and monitored for various lengths of time during which they were provided food and water ad libitum.

Bronchoalveolar Lavage (BAL)

To perform BAL, a 450 mg/kg dose of avertin – diluted from stock in 10% sodium chloride solution – was injected IP to anesthetize the mice. The anesthetized mouse was verified unresponsive to tactile stimulation and the surface of the mouse was treated with a 70% ethanol solution to reduce the possibility of microbial contamination. An incision was made along the ventral midline and blunt dissection used to free the trachea. A 5 mm incision was made in the midline of the trachea and a lavage tube (cannula) was inserted into the trachea and stabilized with 10-0 silk suture material. The end of the cannula outside the trachea contained a luer lock for the attachment of a syringe. PBS (1X, 4°C) was introduced into the lung via the cannula to wash the lung three times – 800 µL for the first wash, and 600 µL for the second and third wash. The recovered lavage volumes were stored on ice, pooled, the total volume measured, and spun down at 1500 rpm for 6 minutes at 4°C. The supernatant was removed, cells suspended in 2 mL ACK lysing buffer (Quality Biological, INC.), incubated

at room temperature for 5 minutes, and then diluted in another 2 mL volume of cold PBS before a second round of centrifugation. (Occasionally two washes with ACK buffer were required to lyse all erythrocytes in bloodier BALs.) The supernatant was removed and the cells suspended in 1 mL 4°C PBS and stored on ice. The number of cells in the BAL was evaluated using light microscopy and a hemacytometer (Hausser Scientific) and the data were expressed as the number of cells per mL of recovered BAL fluid. To obtain differential counts, the cells were diluted to 100,000 cells/-mL and 1 mL was adhered to a glass slide with the aid of cytology funnels (Fisher Scientific) and a cytocentrifuge (CytoSpin, Shandon). Cells were subsequently fixed and stained utilizing Diff-Quik® (Andwin Scientific) and enumerated using light microscopy.

Hemoglobin Assay of BAL Fluid

A hemoglobin assay was used to estimate the quantity of blood that was collected in the BAL. 100 µL of ACK lysing buffer was added to 100 µL of BAL fluid, allowed to sit at room temperature for 5 minutes, centrifuged at 4°C for 6 minutes at 1500 rpm, and 100 µL of supernatant was loaded into a microtiter plate. Absorbance readings at 405 nm were collected using a spectrophotometer (Molecular Devices, LLC). The 1:1 dilution of BAL with ACK buffer was performed to avoid spectrophotometer limitations at high concentrations.

Lung Edema

Whole lungs were weighed immediately after resection (wet weight) at 1, 3, and 5 days post-PPE. Subsequently they were dried in a 65° C incubator for 72 hours and again weighed (dry weight). Wet weights, dry weights, and wet-to-dry ratios were used to comparatively evaluate lung edema in neutrophil-depleted and neutrophil-intact mice.

Lung Histology

Mice were anesthetized with avertin and the trachea was exposed and cannulated as described above. Z-Fix® (Anatech LTD.) was introduced down the cannula at a constant pressure of 30 cm of water for 3 minutes. Fixed lungs were then carefully dissected out of the body cavity to prevent perforation and stored in conical centrifuge tubes containing 35 mL of Z-Fix® for 48 hours before further processing. Random, 2-3 mm square pieces were cut from fixed lungs and imbedded in paraffin blocks. From these blocks five micron sections were cut and stained with hematoxylin and eosin (H&E) for light microscopy and imaging. Lung sections were analyzed to measure the extent of emphysema using the mean airspace chord lengths (L_m) based on calculations described by Knudson and colleagues (62). L_m was calculated using parenchymal volume and alveolar surface area measurements, being directly proportional to volume (V) and inversely proportional to surface area (SA) (62).

Lung Function Analysis

Total Lung Capacity (TLC)

A cannula was inserted into the trachea of anesthetized mice and connected to a constant-flow ventilator. Following a deep inspiration to 30 cmH₂O for five seconds, the mouse was returned to normal ventilation – tidal volume of 0.2 mL of 100% O₂ at a rate of 150 breaths per minute – before degassing of the lungs for 3 minutes. A previously described method for generating quasi-static pressure-volume curves was utilized after degassing (63). To summarize, two sequential airway P-V loops were acquired by the PowerLab digital data-acquisition system beginning at a pressure of 0 cmH₂O and ending with a maximum pressure of 35 cmH₂O. The volume corresponding to a pressure of 35 cmH₂O was defined as TLC. TLC is a useful parameter for detecting emphysematous physiology; high TLC values indicate destruction of lung architecture.

Diffusion Factor of the Lung for Carbon Monoxide (DF_{CO})

Anesthetized, intubated mice were assessed for the extent to which carbon monoxide (CO) diffused from inspired air into the lung parenchyma in relation to an insoluble comparator, neon (Ne). At a constant pressure and volume, diffusion of CO is dependent on alveolar SA, alveolar thickness, blood flow to pulmonary capillaries, and the rate of chemical binding to hemoglobin (64). DF_{CO}, as previously described (REF), involves four gas chromatograph (GC) measurements: concentration of CO in the calibration gas, concentration of CO in the inspired gas, concentration of Ne in the calibration gas, and concentration of Ne in the inspired gas (64). 0.8 mL of calibration

gas – approximately 0.5% CO and 0.5% Ne in composition – is injected using a syringe to inflate the lung, and after 9 seconds the gas is withdrawn, diluted to 2 mL with room air, and analyzed using a GC. When the concentration of CO in the inspired gas is low compared to the calibration gas, the diffusion factor is high, and vice versa. An emphysematous lung has lost surface area for gas exchange and thus has a reduced DF_{CO} .

Neutrophil Depletion

To deplete neutrophils, 500 µg of the Ly6-G-specific antibody clone 1A8 (Bio X Cell, Catalog#: BE0075-1) was administered IP 24 hours prior to IT challenge with 3 U of PPE. To assess the extent and dynamics of neutrophil depletion in the peripheral blood, tail vein blood samples were taken at days -1, 0, 1, 2, 3, 5, 7, and 9 from mice in two experimental groups (n=2). Both groups received antibody on day -1. To determine if PPE had an effect on antibody depletion, one group received PPE on day 0 and the other group received an equivalent volume of PBS. Each blood sample was diluted in 1 mL cold PBS and processed for differential cell counts as outlined above for BAL samples. Additionally, to compare neutrophils in the peripheral blood of 1A8-treated and elastase-only treated mice tail vein blood samples were taken at days -1, 0, 1, 2, 3, 4, and 5. Three mice were randomly assigned to receive 1A8 on day -1 and another three mice received a PBS injection of equivalent volume also on day -1. All mice received PPE on day 0.

To analyze the effects of neutrophil depletion on BAL cell dynamics, antibody or PBS was injected at day -1, PPE administered at day 0, and BAL samples were collected at days 1, 3, and 5 (n=3). To analyze the effects of neutrophil depletion on lung histology, antibody or PBS was injected at day -1, PPE administered at day 0, and lungs were harvested at days 1, 3, 5, and 21 for processing. Histology was repeated four times – once as a stand-alone experiment (n=3) and three times after first performing pulmonary function tests.

To further characterize the effects of depletion, mice were administered antibody or PBS, dosed with PPE 24 hours later, incubated 21 days, after which various parameters of pulmonary function – namely TLC and DF_{CO} – were measured. Pulmonary function testing was performed on three occasions.

Statistical Analysis

Graphpad Prism 4.0a was used for all statistical analysis. An unpaired, two-tailed t test was used to generate p values for all data.

RESULTS

Neutrophil-Intact Response to Elastase Challenge

In the mouse elastase model elastase is administered intratracheally (IT) and is aspirated into the lungs where it presumably exerts its main effect by degrading the elastin component of the extracellular matrix causing a disruption in lung epithelial cell organization and function, which in turn results in a breach of barrier integrity, hemorrhage, and an influx of inflammatory cells. Disruption of this barrier has immediate effects and triggers an acute innate inflammatory response. This acute phase, while measurable and important, only sets the stage for emphysema to develop later – weeks after the initial elastase insult. We first sought to define this acute phase. BAL fluid collected from washing the lung lumen with buffered saline contains cells that reflect the inflammatory state of the lung and is useful for our analyses.

Comparing C57BL/6 and BALB/c Mice

Bronchoalveolar lavage (BAL) cell dynamics in response to IT elastase had been previously followed over five days in C57BL/6 mice. Recently, it was determined that BALB/c mice produced a more pronounced and recognizable emphysema phenotype compared to C57BL/6 mice. In addition, the increased sensitivity of the BALB/c strain required an adjustment to the amount of elastase administered from 6U to 3U in order to produce the appropriate level of emphysema. Since there were no data available on BALB/c mice challenged with 3U of elastase, experiments were designed to define the

cellular dynamics in the lungs. The experiment was designed to compare BAL cell dynamics in C57BL/6 and BALB/c mice over the acute time frame post-PPE exposure.

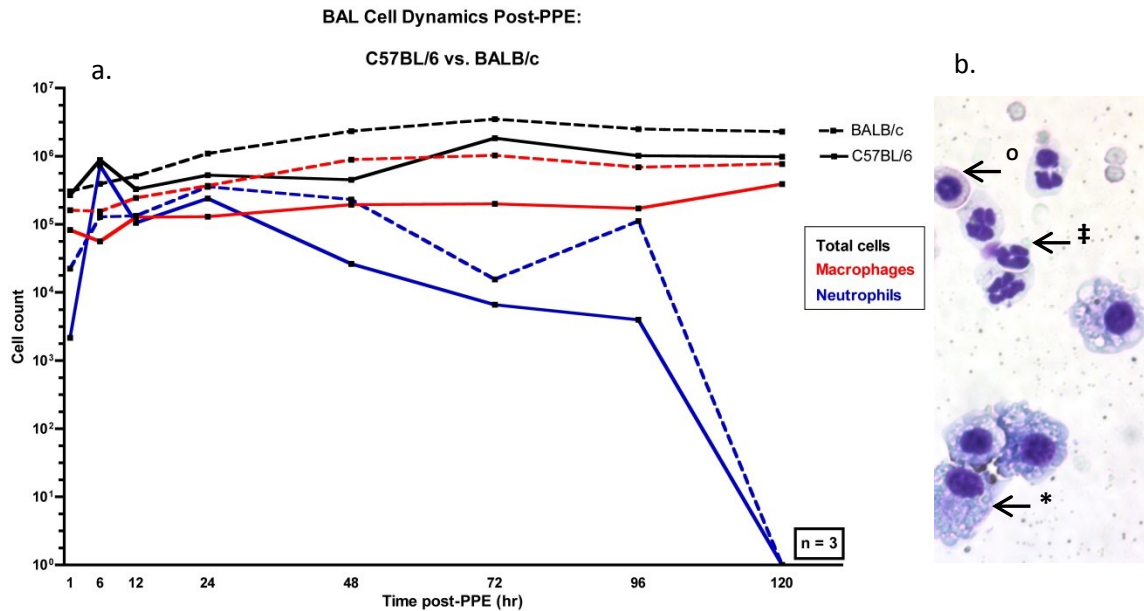


Figure 5: (a) C57BL/6 and BALB/c mice have similar BAL cell dynamics in response to IT elastase. BAL was collected at 1, 6, 12, 24, 48, 72, 96, and 120 hours post-PPE, and each point represents the average of three mice. (b) Representative image of BAL-derived macrophages*, monocytes^o, and neutrophils[‡] obtained from a BALB/c mouse at 24 hours post-elastase with a 40X objective lens.

In general, the cellular dynamics of the BALs from BALB/c and C57BL/6 mice were comparable (Figure 5a). A notable difference was the more rapid and higher neutrophil influx in C57BL/6 mice as compared to the slower and smaller BALB/c influx within the first few hours after elastase administration. At later time points, the neutrophil numbers and kinetics were essentially the same between the two strains. Stained cytospin preparations of cells isolated in the BAL at 24 hours post-elastase showed neutrophils, monocytes, and macrophages with standard morphology (Figure 5b).

It was concluded that the cellular dynamics in response to elastase administration was not significantly different between BALB/c and C57BL/6. All proceeding experiments utilized BALB/c mice.

Cell Dynamics – Characterizing the BAL Fluid

Repeated measures of the BAL cell dynamics in BALB/c mice in response to elastase challenge were recorded over a five day period (Figure 6) and served as a useful baseline comparator to guide the subsequent 1A8 neutrophil depletion experiments.

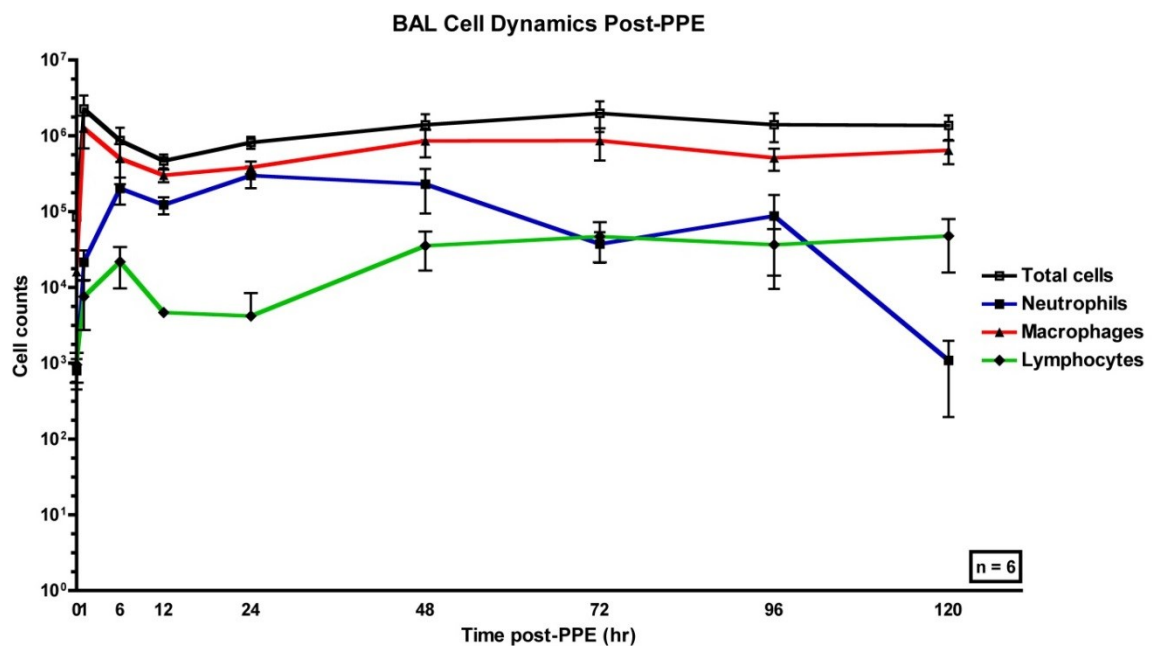


Figure 6: BAL cellular profile in BALB/c mice at 1, 6, 12, 24, 48, 72, 96, and 120 hours post-PPE. Neutrophils, macrophages, lymphocytes, and total cells were counted for every BAL sample and each point represents the average of six mice.

Neutrophil Dynamics

At baseline, an average of 793 neutrophils were present in the BAL (range: 0 – 1938) (SEM: 345). The infiltration of neutrophils into the lungs 1 hour after PPE administration was significant ($p = 0.0497$), with counts rising to 21,480 neutrophils (range: 0 – 57,267) (SEM: 9,263). Neutrophils peaked at 24 hours post-PPE at an average of 298,530 cells (range: 47,075 – 532,904) (SEM: 94,804). At their peak, neutrophils comprised 24.97% of all BAL cells. Neutrophils began to decline at 48 hours, and returned to near pre-challenge levels by 120 hours. (Figure 6)

Macrophage and Monocyte Dynamics

At baseline, an average of 16,080 mononuclear cells were present in the BAL (range: 9,614 – 21,563) (SEM: 1,748). An unexplained spike in macrophages was seen at 1 hour post-PPE, but this spike was not significant and is likely due to sampling artifact, reflected in the large standard error at this time point (SEM: 582,300). While no significant differences were found between adjacent time points, the general trend suggests that mononuclear cells do increase in the lung lumen in response to IT elastase, plateau after 48 hours (887,134 macrophages, or 49% of BAL cells), and by 120 hours their numbers still hold relatively steady (767,913 macrophages, or 50% of BAL cells) (Figure 6). Variation between mice is responsible for the lack of statistically significant difference, and is a recurring theme in many of the cell dynamics results.

Lung mononuclear cell dynamics are very different from neutrophil dynamics. Their influx is not only delayed but also substantial – macrophages and monocytes make up the largest fraction of the BAL cellular response to IT elastase.

Lymphocyte Dynamics

Lymphocytes made up a small percentage of BAL cells at any given time point (Figure 6). The jump in lymphocytes observed at 48 hours and subsequent plateau, while not statistically significant ($p = 0.1804$), does parallel macrophage dynamics. Additionally, the increase in lymphocytes from baseline to 1 hour post-PPE was not significant ($p = 0.0795$) and is likely due to blood leaking into the lumen.

Total Cell Count Dynamics

Without elastase, an average of 85,000 cells are recovered from BAL fluid (range: 65,000 – 115,000) (SEM: 8,165). 1 hour post-PPE this number jumps to over 2.25×10^6 cells, albeit the standard error associated with this number is enormous (SEM: 1,134,760). This suggests one of two things: the variability between mice is huge, or the method of quantifying cells is unreliable. BAL samples collected at 12, 24, 96, and 120 hours post-PPE had significantly more cells compared to baseline, but at 1, 6, 48, and 72 hours cells were not significantly elevated compared to baseline. This speaks more to the variation between mice at a given time point rather than being a true descriptor of post-PPE cell dynamics. The trend towards elevated total cell counts is evident at *all* acute time points post-PPE (Figure 6).

Blood in the BAL

Blood in the BAL fluid is evidence of lung damage, and thus is useful for characterizing animal dosing, assessing PPE activity, indirectly measuring the extent and duration of epithelial and endothelial cell damage, and estimating the time scale for acute resolution. Hemoglobin is evident in the BAL fluid as early as 1 hour post-PPE (Figure 7). Without an elastase insult, an average absorbance of 0.115 was recorded (n=6) (range: 0.110 – 0.123) (SEM: 0.002). Absorbance at 405 nm was significantly elevated compared to baseline in samples from all time points post-PPE, proving the potency of our enzyme. Parallel to the influx of neutrophils, blood in the BAL peaked between 24 and 48 hours after which it progressively declines. An interesting observation was that while variation did exist between mice, later time points exhibited less variation than earlier time points.

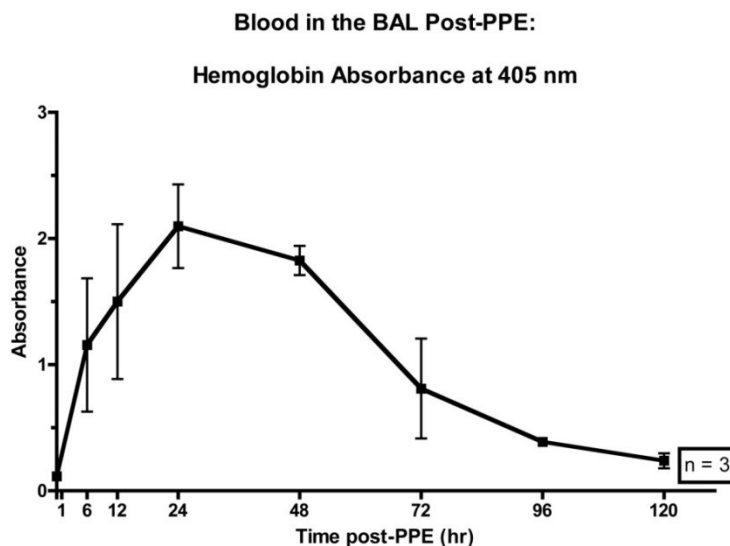
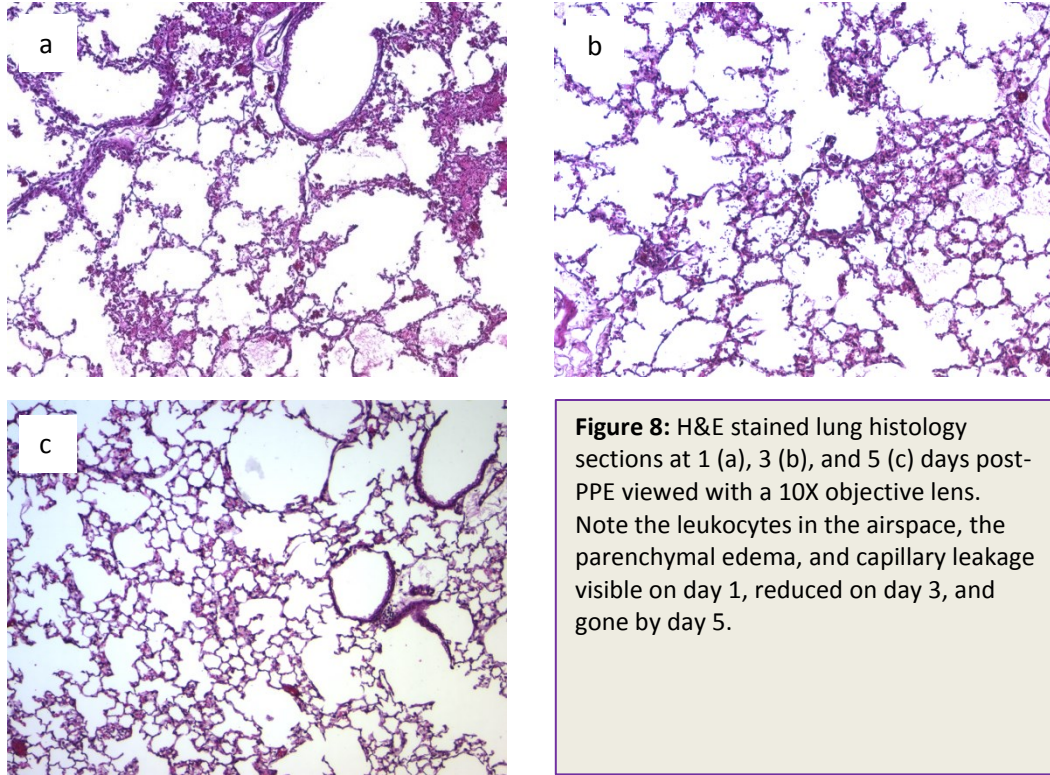


Figure 7: Absorbance at 405 nm was used as a marker for hemoglobin in BAL samples taken at 1, 6, 12, 24, 48, 72, 96, and 120 hours. Each point represents the average of three mice.



Lung Histology – Defining the Extent of Alveolar Destruction

Lung histology was examined at 0, 1, 3, and 5 days post-PPE, and upon gross inspection intense hemorrhage was observed on day 1 (Figure 8a). Hemorrhage was less evident on day 3 (Figure 8b) and undetectable by day 5 (Figure 8c) post-PPE. The 1, 3, and 5 day post-PPE pattern for lung hemorrhage seen in histology sections is also reflected in the 405 absorbance of BAL samples (Figure 7). Histology offers direct evidence of the injury response in the context of alveolar architecture. No quantification of lung damage was done for these histology sections as emphysema cannot be measured at acute time points.

Neutrophil-Depleted Response to Elastase Challenge

After measuring the acute dynamics of cells and blood in the BAL fluid after elastase challenge, the next aim was to deplete neutrophils during their active window and look to see how this affected the development of emphysema. Emphysema is a chronic disease and mice in these experiments require longer incubation post-PPE. In the elastase model of emphysema, 21 days is sufficient time to see emphysematous changes in lung histology sections, and because lung physiology is a reporter of lung architecture 21 days is also suitable for measuring lung function.

Systemic Depletion of Neutrophils – Action of 1A8 Antibody

The first task was to verify that 1A8 was both specific and effective in depleting neutrophils, and to identify its length of action when administered by IP injection.

In the peripheral blood, neutrophils were successfully and significantly depleted in 1A8-treated mice 24 hours after antibody injection compared to controls ($p = 0.0004$) (Figure 9). In elastase-only treated mice, the neutrophil fraction was maintained at a relatively constant 24% of blood leukocytes throughout the six day period. However, blood taken 1 day after 1A8 antibody administration was virtually devoid of neutrophils and this depletion persisted 6 days after antibody was administered, or 5 days post-PPE, at which point we ceased sampling. Evidence of rebound was observed but the repopulation of neutrophils appeared to be a slow process.

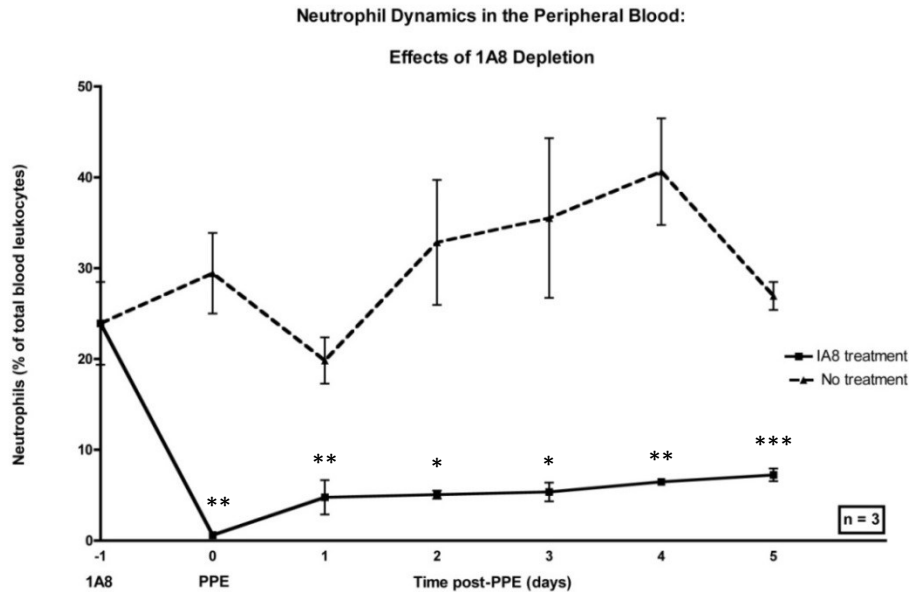


Figure 9: A single 500 μ g dose of 1A8 antibody injected IP 24 hours prior to elastase administration is enough to sufficiently deplete neutrophils for days in the peripheral blood. 16 μ L of tail vein blood was taken from each mouse at days -1, 0, 1, 2, 3, 4, and 5 post-PPE. Each point represents the average of three mice. At all time points after 1A8 administration, neutrophils were significantly depleted in the peripheral blood of mice who received antibody when compared to mice who did not receive antibody. Testing to determine statistical significance between treatment groups at each time point was conducted using an unpaired two-tailed t test. ($p < .05^*$) ($p < .01^{**}$) ($p < .001^{***}$)

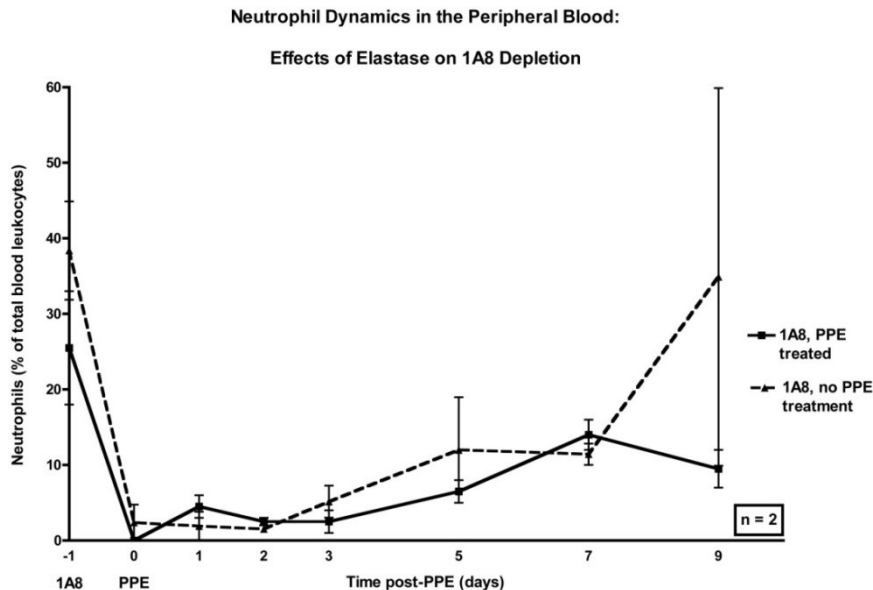


Figure 10: IT elastase administration 24 hours after a single 500 μ g injection of 1A8 antibody does not affect 1A8-mediated systemic neutrophil depletion. 16 μ L of tail vein blood was taken from each mouse at days -1, 0, 1, 2, 3, 5, 7, and 9 post-PPE. Each point represents the average of two mice.

Next we wanted to determine if IT administration of elastase had any impact on the efficacy of the 1A8 antibody in depleting neutrophils (Figure 10). Peripheral blood neutrophil levels over time measured in tail vein blood from animals treated with elastase 24 hours after administration of 1A8 antibody were not significantly different from the animals that received only 1A8 treatment.

Cell Dynamics – Effects of Neutrophil Depletion on the BAL Fluid

Figure 11 combines results for neutrophil (Figure 12), macrophage (Figure 13), and lymphocyte (Figure 14) BAL dynamics for comparison both between cell types and between treatment groups. 1A8 alters neutrophil dynamics in the five day period following elastase challenge, but not macrophage or lymphocyte dynamics .

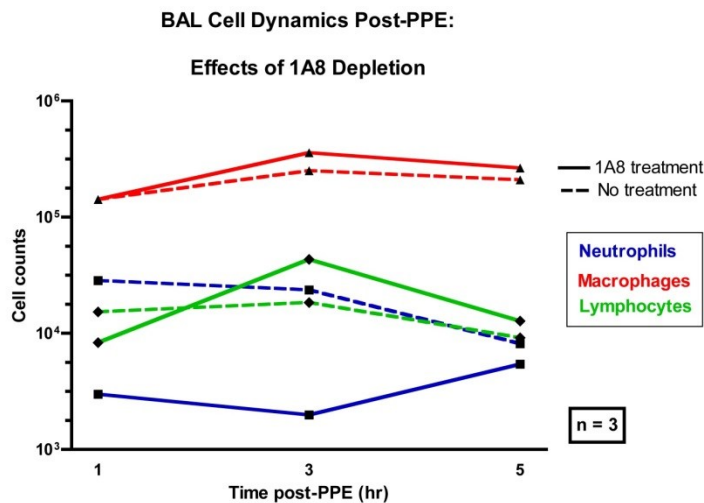


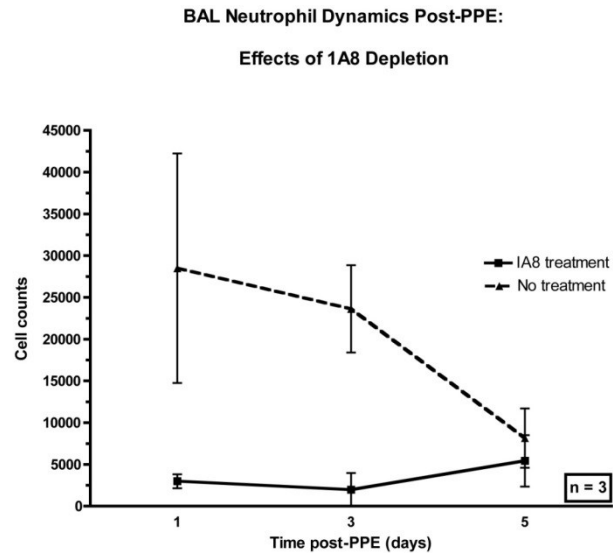
Figure 11: A 500 µg IP injection of 1A8 24 hours prior to IT elastase challenge affects neutrophil dynamics in the BAL, but not macrophage or lymphocyte dynamics. BAL samples were taken at days 1, 3, and 5 post-PPE. Each point represents the average of three mice.

Neutrophil Dynamics

Neutrophil depletion in the peripheral circulation in response to 1A8 injection was paralleled by a lack of neutrophil infiltrate in the lungs (Figure 12). BALs from

neutrophil-depleted mice had an average of 2,607 neutrophils on day 1 post-PPE (SEM: 621), versus 28,498 neutrophils in BAL samples from elastase-only treated mice (SEM: 13,740). While not significant due to variation between mice ($p = 0.1330$), this trend reflects the previously described systemic depletion.

Figure 12: Systemic neutrophil depletion with 1A8 antibody prevents the post-PPE neutrophil influx into the lung. 1A8 was administered 24 hours before elastase challenge, and BAL samples were taken at days 1, 3, and 5 post-PPE. Each point represents the average of three mice.



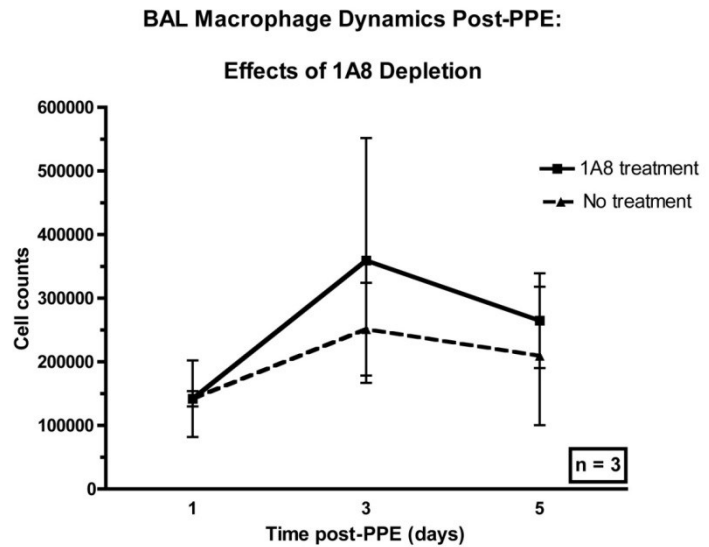
By day 5 elastase-only treated mice had neutrophil levels comparable to those of 1A8-treated mice, leading us to conclude that neutrophils do not rebound within the window of neutrophil influx recorded after elastase challenge (Figure 6). Importantly, this shows that 1A8 treatment alters the acute immune response to IT elastase by removing the key cell type in the protease-antiprotease hypothesis from the lung environment.

In this experiment neutrophil counts from the BAL fluid of elastase-only treated mice were dramatically lower (28,498) than those from previous experiments (298,530). This ten-fold difference was due to fewer total cells being isolated from the BAL fluid and not from the reported percentage of neutrophils, which remain quite comparable.

This reduction in total BAL cells also affects macrophage and lymphocyte numbers, which will be presented next.

Macrophage and Monocyte Dynamics

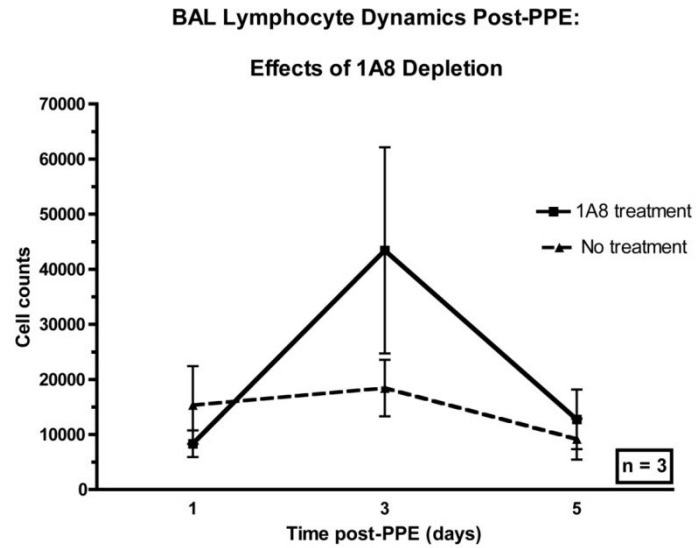
Figure 13: Systemic neutrophil depletion with 1A8 antibody does not significantly affect the macrophage influx into the lungs at day 1, 3, or 5 post-PPE. 1A8 was administered 24 hours before elastase challenge. Each point represents the average of three mice.



There was no significant difference between macrophage and monocyte numbers in the BAL fluid of 1A8-treated versus elastase-only treated mice at any time point post-PPE (Figure 13). Mononuclear cell numbers for 1A8-treated mice were 141,993 (day 1), 359,339 (day 3), and 264,793 (day 5). The corresponding numbers for elastase-only treated mice were 141,992 (day 1), 251,348 (day 3), and 209,299 (day 5). When mononuclear cells are reported as a percent of BAL cells there is no significant difference between experiments (data not shown). Importantly, the trend that macrophages and monocytes peak between 48 and 72 hours, and do not efflux by day 5 is still there.

Lymphocyte Dynamics

Figure 14: Systemic neutrophil depletion with 1A8 antibody does not affect lymphocyte dynamics in the BAL at day 1, 3, or 5 post-PPE. 1A8 was administered 24 hours before elastase challenge. Each point represents the average of three mice.



There was no significant difference in BAL lymphocyte counts between 1A8-treated and elastase-only treated mice (Figure 14). Lymphocyte numbers for 1A8-treated mice were 8,326 (day 1 post-PPE), 43,421 (day 3), and 12,747 (day 5) (n=3). The corresponding numbers for elastase-only treated mice were 15,337 (day 1), 18,415 (day 3), and 9,170 (day 5) (n=3). This trend agrees with lymphocyte results from the first experiment.

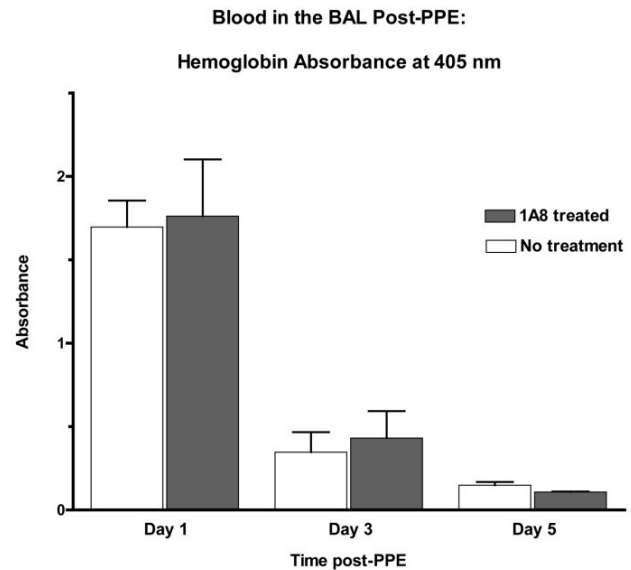
Total Cell Count Dynamics

Cell counts obtained from elastase-only treated mice on days 1, 3, and 5 post-PPE were 224,800, 356,700, and 258,300 respectively (counts represent the average of three mice). Cell counts of mice that received 1A8 antibody (day 1: 313,000, day 3: 613,300, day 5: 338,300) were not significantly different from elastase-only treated mice on any day. We conclude that depleting neutrophils does not appreciably change the

magnitude of the cellular influx in response to IT elastase. This seemingly counterintuitive result will be addressed in the Discussion section.

Blood in the BAL

Figure 15: Hemoglobin in the BAL. A 500 µg injection of 1A8 antibody 24 hours prior to elastase challenge had no significant effect on lung hemorrhage as reported by 405 absorbance. The BALs of elastase-only treated and 1A8-treated mice were compared at days 1, 3, and 5 post-PPE. (n = 3)



Blood in the BAL fluid as measured by the hemoglobin content of the BAL was not significantly different between treatment groups at any time point post-PPE (Figure 15). The observed trend followed an expected pattern – bloody BAL samples at early time points post-PPE that clear as time progresses, reaching baseline blood BAL levels by day 5. This result suggests that neutrophil depletion does not significantly affect the level of acute damage done by elastase that leads to hemorrhage or the time course for repair of the damage.

Lung Edema – Effects of Neutrophil Depletion on Wet-to-Dry Ratios

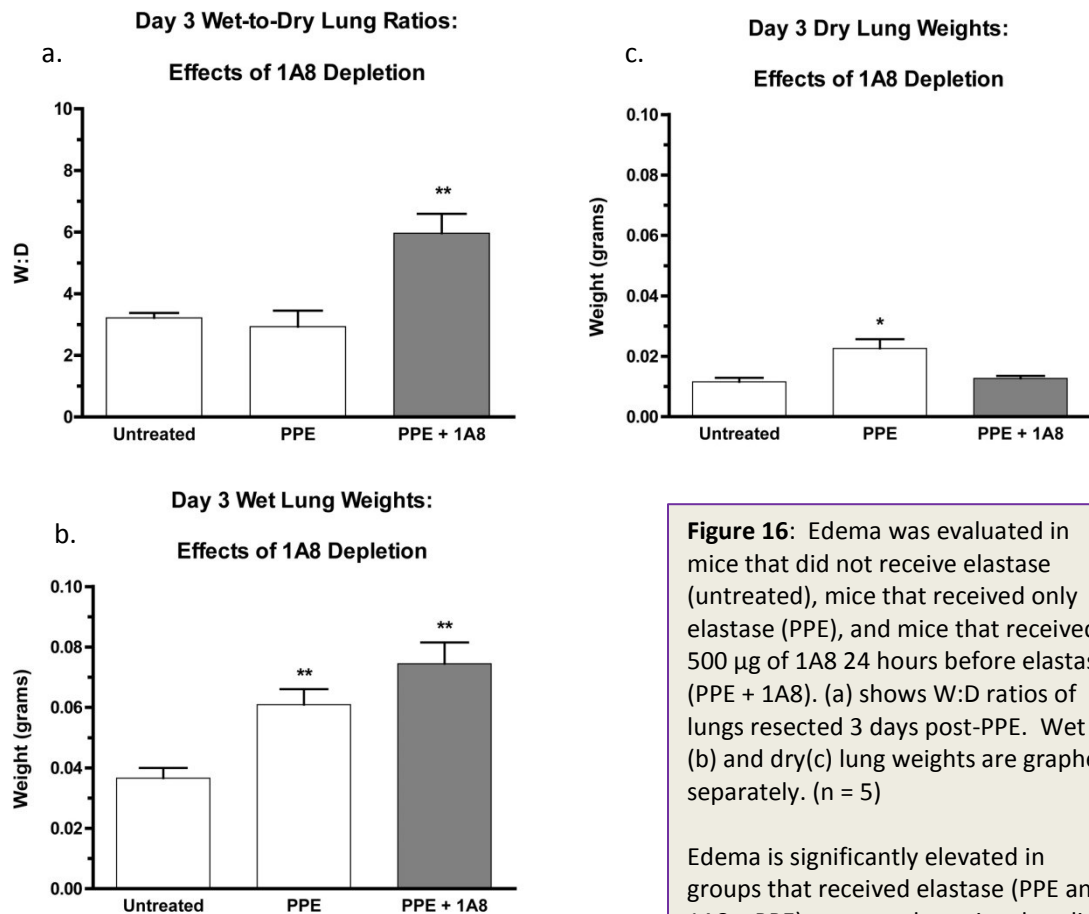


Figure 16: Edema was evaluated in mice that did not receive elastase (untreated), mice that received only elastase (PPE), and mice that received 500 µg of 1A8 24 hours before elastase (PPE + 1A8). (a) shows W:D ratios of lungs resected 3 days post-PPE. Wet (b) and dry(c) lung weights are graphed separately. (n = 5)

Edema is significantly elevated in groups that received elastase (PPE and 1A8 + PPE) compared to mice that did not receive elastase (b). Neutrophil depletion does not significantly affect lung edema after elastase challenge (b).

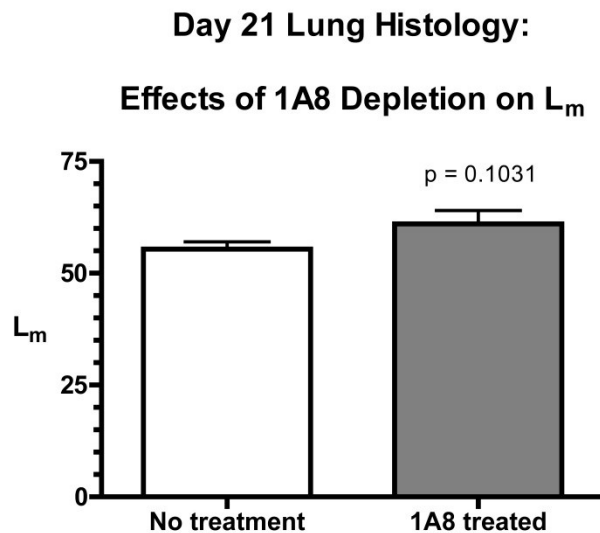
Edema is an indirect measure of acute

damage and the wet-to-dry (W:D) ratio is a common way to quantify it. In the lungs, elastase-mediated damage results in an influx of plasma (edema) and other blood constituents, both of which are reflected in an increase in wet lung weight, and drying the lung removes the weight due to fluid accumulation. Copious edema elevates the W:D ratio; and thus, similar to blood in the BAL, W:D lung ratios can be used to report the extent of acute elastase-mediated damage. At day 3 post-challenge, the 1A8-

treated mice had significantly higher W:D ratios when compared to either the PPE ($p = 0.0118$) or control group ($p = 0.0059$) (Figure 16a). This result was not consistent with the other measure of hemorrhage (hemoglobin, Figure 15), which suggested that there was not a difference in the level of damage. In addition, unexpectedly, there was no significant difference between the PPE group – who received only elastase – and untreated mice – who received no elastase. To address these inconsistencies, we considered the wet and dry lung weights separately. After controlling for mouse weight, PPE mice were observed to have both heavier wet (Figure 16b) and dry (Figure 16c) lungs. This suggests that the W:D ratio is not a suitable measure of lung edema at day 3 following IT elastase.

Lung Histology – Effects of Neutrophil Depletion on Alveolar Destruction

Figure 17: Neutrophil depletion with a single 500 μg injection of 1A8 antibody 24 hours prior to elastase challenge had no significant effect on airspace size 21 days post-PPE. L_m values, calculated from formalin-fixed H&E-stained histology sections, of elastase-only treated mice ($n = 24$) were not significantly different from those of 1A8-treated mice ($n = 23$). The number of mice per group has been pooled from five replicates.



To assess the impact that neutrophil depletion had on the development of emphysema in BALB/c mice, lungs were harvested from neutrophil-depleted and control

animals at day 21 post-elastase and assessed histologically for the extent of alveolar damage. In addition to visual inspection of the sections, mean chord lengths (L_m) were measured. Combining the data from five independent replicate experiments, twenty-four elastase-only treated mice had an average L_m score of 55.40 (range: 35.97 – 74.56) (SEM: 1.645) and twenty-three neutrophil-depleted mice had an average score of 61.04 (range: 41.32 – 91.79) (SEM: 3.010) (Figure 17). This difference in mean airspace chord length did not reach statistical significance ($p = 0.1031$); thus, by this measure, we concluded that neutrophils do not play a significant role in the regulation of elastase-induced emphysema (Figure 17).

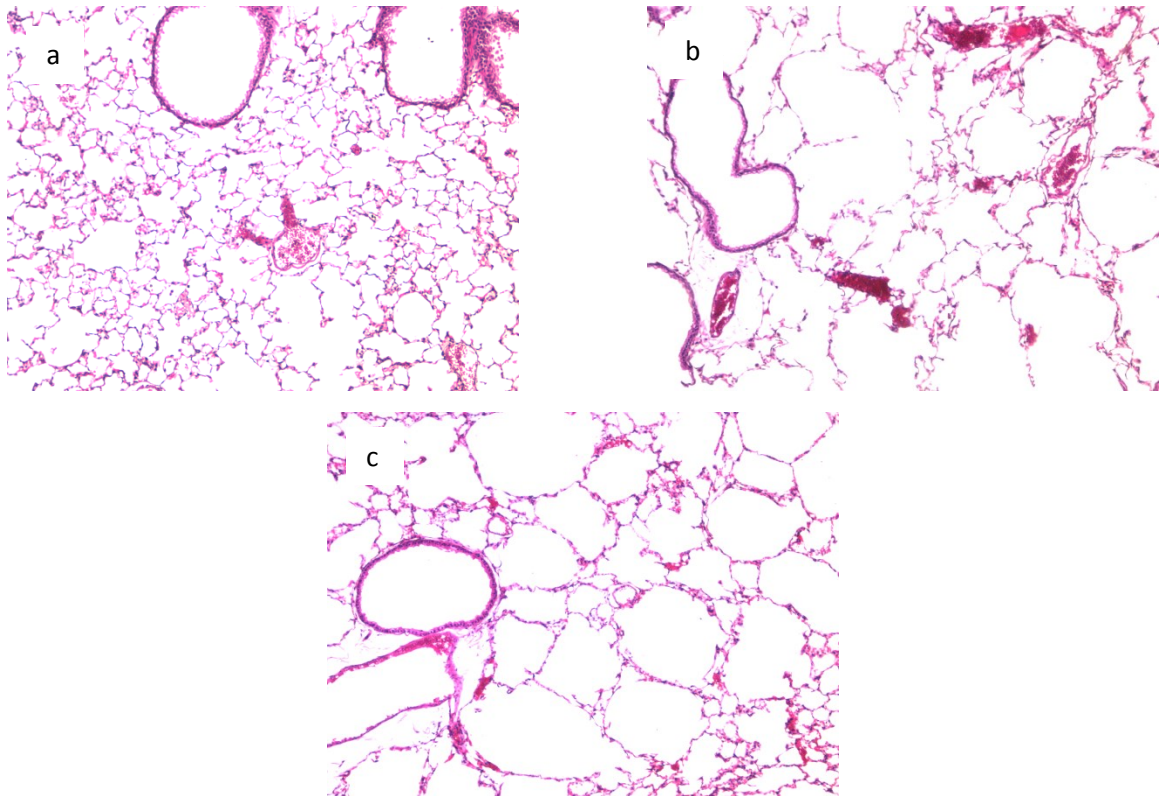
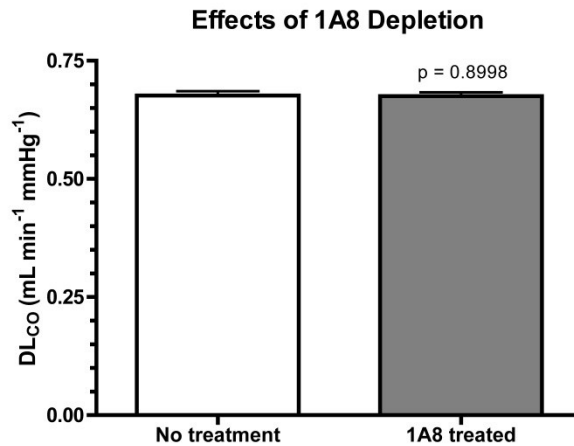


Figure 18: Representative samples of H&E stained lung histology sections viewed with a 10X objective lens and taken from an untreated mouse (a), an elastase-only treated mouse (b), and an elastase and 1A8-treated mouse (c). Lungs were resected at 21 days post-PPE. Note differences in the volume-to-surface area ratio. (a) has a low L_m value, while (b) and (c) have high L_m values. This underlying pathology is responsible for the clinical presentation of emphysema.

Lung Function – Effects of Neutrophil Depletion on TLC and DF_{CO}

Figure 19: Neutrophil depletion with a single 500 μg injection of 1A8 antibody 24 hours prior to elastase challenge had no significant effect on lung function 21 days post-PPE. DF_{CO} values of elastase-only treated mice ($n = 20$) were not significantly different from those of 1A8-treated mice ($n = 21$). The number of mice per group has been pooled from four replicates.

Day 21 Diffusing Capacity of the Lung for Carbon Monoxide:



While the presence of neutrophils did not appear to influence the elastase-induced lung damage, it is possible that it does impact lung function. DF_{CO} measures the gas exchange capacity of the lung – low values indicate impeded gas exchange and can be due to decreased surface area, as in emphysema, or interstitial thickening, as in asthma. DF_{CO} values of 1A8-treated mice 21 days post-PPE were not significantly different from the values of elastase-only control mice (Figure 19).

Day 21 Total Lung Capacity:

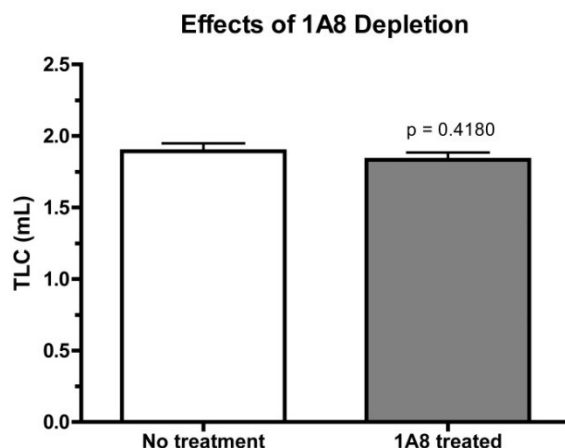


Figure 20: Neutrophil depletion with a single 500 μg injection of 1A8 antibody 24 hours prior to elastase challenge had no significant effect on lung volume 21 days post-PPE. TLC values of elastase-only treated mice ($n = 19$) were not significantly different from those of 1A8-treated mice ($n = 21$). The number of mice per group has been pooled from four replicates.

Another measure of lung function is total lung capacity (TLC). TLC is elevated in emphysema due to parenchymal destruction and it correlates with the increasing airspace seen in lung histology sections. TLC measurements were not significantly different between 1A8-treated mice and control animals ($p = 0.4180$) (Figure 20). This agrees with our previous L_m data and supports the conclusion that neutrophil depletion had no impact on the level of elastase-induced emphysema.

DISCUSSION

Our experiments were designed to test the hypothesis that the early influx of neutrophils into the lungs (A) contributes to the resolution of acute elastase-mediated injury and (B) affords protection to the lungs from the progressive alveolar destruction that is associated with emphysema.

Our experiments in BALB/c mice are the first report of neutrophil, macrophage, lymphocyte, and total cell BAL dynamics over early and late time points in the acute response to three units of IT elastase. The early and transitory presence of neutrophils was confirmed, and contrasted with the delayed and prolonged presence of macrophages. This is not significantly different from the cellular dynamics observed in C57BL/6 mice receiving either 6U or 3U of elastase. Neutrophils arrive rapidly within one hour post-PPE, peak between 24 and 48 hours, begin to fall by 72 hours, and are at pre-challenge levels five days after the inciting stimulus. These data established the time frame that was critical for testing the hypothesis that neutrophil recruitment to the lungs impacts the short-term and long-term consequences of elastase-induced pulmonary damage.

We found that a single 500 µg dose of the anti-Ly6G antibody, 1A8, 24 hours prior to elastase administration was sufficient to sustain a profound neutropenia in the peripheral blood over the entire dynamic period post-elastase challenge when control animals see a significant influx of neutrophils. The test and control animals tolerated

1A8 treatment well suggesting that transient neutrophil depletion had minimal impact on overall health.

Based on the role that neutrophils play in wound repair and tissue remodeling in other tissues (25), we hypothesized that the presence of neutrophils after elastase administration would play a role in limiting the extent of damage and promote repair. The observation that the amount of blood in the BAL fluid (used here as a marker of acute damage to the alveolar epithelium and endothelium resulting in hemorrhage) as well as the duration of blood in the BAL (used as a surrogate for how rapidly the damage was repaired) did not change significantly in response to neutrophil depletion suggested one of two things. First, in our model the high dose of elastase administered to each mouse might cause damage so extensive and immediate in nature as to mask any acute amelioration from neutrophils. Second, it is possible that these ameliorating effects from neutrophils might only manifest later during the chronic resolution phase. The conclusion that neutrophil depletion did not significantly alter the level of elastase-induced damage was also supported by the lung W:D data. Additional studies are needed to define the roles of neutrophils in the resolution of acute tissue damage in the lungs.

It was anticipated that as a result of neutrophil depletion there would be an alteration in the number, dynamics and composition of the cellular infiltrate associated with elastase-induced damage in the lungs. Unexpectedly, the total number of cells measured in the BAL in response to elastase challenge as well as the composition of the cellular infiltrate were not significantly affected by neutrophil-depletion. The reasons

for this unexpected outcome are not clear. One factor that influenced the results was a high level of experimental variation in cell counts. High standard errors were obtained for total cell counts *within* experiments; and additionally, significant differences existed in cell counts *between* experiments. The total number of cells isolated from BAL samples was reduced in the antibody depletion experiment. Cell counts obtained from elastase-only treated mice on days 1, 3, and 5 were 224,800, 356,700, and 258,300 respectively. Day 1, 3, and 5 counts from earlier experiments (see Figure 6) were 817,500, 1,983,333, and 1,367,500 respectively. The reasons for this variation in cell counts is unclear. Variability in the BAL cellular response to IT elastase could be due to variable dosing, variable delivery to the various lobes of the lungs, variable enzymatic activity, and/or natural variations between mice. However, because no significant difference in total cell counts was observed between neutrophil-depleted and neutrophil-intact mice after antibody-mediated neutrophil depletion we conclude that the presence of neutrophils in damaged lung tissues does not appreciably influence the magnitude or the cellular composition of the cellular influx in response to IT elastase.

This conclusion seems at odds with our understanding of the consequences of treatment with the neutrophil-depleting 1A8 antibody. 1A8 depletes a cell type common in post-PPE BAL samples, which should affect the total cell counts. One interpretation of this is that variation between mice keeps the small reduction in total cells due to 1A8 statistically insignificant. Recall from earlier results that neutrophils are not the primary cell type isolated from BAL fluid – macrophages are. Because total BAL

cell counts are used to calculate absolute neutrophil, macrophage, and lymphocyte numbers, total cell variability is reflected in all cell quantification data.

We have shown that macrophage numbers increase in the lungs – whether due to local proliferation or systemic recruitment – in response to IT elastase, peak around 48 to 72 hours post-PPE and remain elevated throughout the acute 5-day period. Of interest is the observation that neutrophil depletion does not significantly affect macrophage dynamics, offering the possibility that recruitment and signaling between neutrophils and macrophages in the elastase model is largely one-way. However, until the effector profile of the lung macrophage population is examined in both a neutrophil-intact and a neutrophil-depleted response to IT elastase the possibility remains that neutrophil depletion protects mice from emphysema through its indirect impact on macrophages.

Lymphocytes are relatively scarce in the lung at all time points post-PPE suggesting any lymphocyte involvement in emphysema would occur later outside of the acute response. This agrees with our understanding of the biology of lymphocytes and their roles in adaptive immune response.

The main hypothesis of this study that neutrophils are protective and that neutrophil depletion would result in an increase in the level of emphysema was not supported by the data. The results compiled from five independent experimental replicate cohorts that measured mean airspace chord length (L_m), the diffusion coefficient (DF_{CO}), and TLC demonstrated that neutrophil depletion did not significantly affect the degree of elastase-induced emphysema as measured at day 21. These results

prompt a reevaluation of the possible roles that neutrophils play in the induction and progression of emphysema.

The phenotype associated with an alpha 1-antitrypsin deficiency and the impressive degree to which neutrophil elastase KO mice are protected from emphysema have been used to justify the elastase model (56). But problems with the elastase model are evident – there is no reproduction of the cigarette smoke exposure and hypoxic conditions. It was previously discussed that cigarette smoke and hypoxia alter the phenotype of effector cells (2) (37). Cigarette smoke likely plays a role not accounted for in the elastase model of emphysema; and thus extrapolation of research in the elastase model to human emphysema is not straightforward.

Remembering that cigarette smoke is the strongest risk factor for the development of emphysema in humans, that the lungs of smokers are exposed to cigarette smoke often many times in one day, and that cigarette smoke interferes with the normal apoptosis and clearance of neutrophils from sites of sterile damage (2) it is reasonable to question whether such a “neutrophil window” exists in emphysema patients. These factors would only increase the amount of time neutrophils are activated in the lung. If no significant differences in alveolar destruction (L_m) and lung function (TLC and DF_{CO}) are seen when neutrophils are present for a short time (i.e. elastase model), it is possible that neutrophils play a bigger role under conditions of chronic exposure (i.e. long-term smokers).

Elevated neutrophil counts in the airway and sputum correlate with the rate of declining lung function and the extent of peripheral airway dysfunction, respectively (2). Despite overwhelming evidence for neutrophilia and neutrophil elastase in smoker sputum and bronchioles (2), it is suspect that neutrophils are not particularly prevalent in biopsies of the lung parenchyma – where the alveolar destruction actually occurs (4). Also suspect is the transitory presence of neutrophils relative to other effector cells. If we consider Occam's razor and the law of parsimony, it is most likely that the effector mechanisms responsible for a chronic disease like emphysema should be present throughout disease initiation, progression, and should disappear if and when the disease resolves or progression slows.

Ultimately, the experimental evidence presented here using the BALB/c model indicates neutrophils do not significantly contribute to the development of emphysema. Measurements of alveolar destruction, functional surface area for gas exchange, and lung volume all show that neutrophil depletion is not protective. Our findings give credence to theories supporting the involvement of other immune cells. Many of these theories were discussed in the introduction. In brief, they evidenced the potential importance of macrophages and the prospect of autoimmune involvement. A 1999 neutrophil depletion study in cigarette smoked rats also concluded that neutrophils were not important in the pathogenesis of emphysema (61). Clinical observations that many antiprotease drugs – some targeting neutrophil elastase – have failed to stem the progression of emphysema agree with our findings. Most importantly, one interpretation of our data puts a dent in the prevailing protease-antiprotease hypothesis

– emphysema is not a disease of renegade neutrophil elastase. Another interpretation is that the BALB/c elastase model is not suitable for studying emphysema. Neutrophil depletion in other animal models of emphysema is certainly justified and will help put these results in perspective.

Clearly, emphysema is a complex, multifactorial disease and there is much work still to be done. In conclusion, this research contributes to the field in a novel way by directly showing no diminution of disease when neutrophils are systemically depleted at acute time points relative to sterile airway insult in an elastase model of emphysema.

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Catherine C Bradley

Date of birth: June 29, 1990

Place of birth: Houston, TX

Cell phone: 281-770-6859

Email: cbradley@jhsph.edu

Address (current): 1101 N Calvert St., Apt. 1801

Baltimore, MD 21202

Education

Johns Hopkins Bloomberg School of Public Health – Baltimore, MD *Aug '12 – Present*

GPA: 3.76

Expected Graduation May 2014

Masters of Science (ScM) in Molecular Microbiology and Immunology

Thesis Research: The dynamics and role of neutrophils in an elastase
model of emphysema

Principal Investigator and Thesis Advisor: Alan Scott, Ph.D.

Rotation Research: Investigating novel metabolism-based compounds for
the treatment of epilepsy

Principal Investigator: Adam Hartman, M.D.

The Certificate in Vaccine Science and Policy, Dept. of International Health

Texas A&M University (TAMU) – College Station, TX

GPA: 3.615

Aug '08 – Aug '12

Graduated Aug 2012

Bachelor of Science in Biomedical Science (BIMS)

Work Experience

TAMU Dept. of Veterinary Pathobiology – College Station, TX

Aug '11 – Aug '12

Microbiology Lab Teaching Assistant

- Supervise students and enforce lab safety
- Teach students the theory behind and techniques associated with biochemical tests specific for bacteria

TAMU Student Learning Center – College Station, TX

Jan '10 – May '12

Organic chemistry, Zoology Supplemental Instruction (SI) Leader

- Lead and organize group study sessions
- Prepare worksheets and practice problems
- Review material with students who elect to attend
- Introduce new study habits and learning methodology to students

Volunteer Work

Johns Hopkins Hospital – Baltimore, MD

July '13 – Dec '13

Student Volunteer

- shadow David Feller-Kopman, M.D. to gain a better knowledge of interventional pulmonology, teaching hospital operations, and emphysema – the subject of my Master's thesis work

Chemistry Road Show (organized through TAMU) – TX

Nov '11

Chemistry Road Show Assistant

- travel to local schools and assist James Pennington, Ph.D. in chemistry demonstrations designed to stimulate an interest in science among students

OB/GYN Affiliates of Katy – Katy, TX

May '11 – Aug '11

Student Volunteer

- shadow Selina Lin, M.D. to gain a better knowledge of common OB/GYN health practices, doctor-patient relations, and the business side of medicine
- take patient vital signs

College Station Medical Center – College Station, TX

Aug '10 – May '11

Aggieland Volunteer

- assist the nursing staff of the cardiac care unit with filing, other secretarial work, and nonmedical patient care
- answer call phones that connect patient rooms to the nursing station

Activities

TAMU Dept. of Veterinary Pathobiology – College Station, TX

May '12 – Aug '12

Student Researcher

- Research: Assembly and sequencing of the Avian Bornavirus genome
- Principal Investigator: Susan Payne, Ph.D.

BIMS Directed Studies – College Station, TX

Jan '11 – May '11

Biomedical Microbiology Lab 485

- one of a small number of students selected to train as future microbiology lab teaching assistants
- assist in the demonstration of laboratory techniques and help teach the conceptual basis of laboratory procedures

Research Skills

PCR

Bacterial culture and cloning

Western blotting

Primary cell isolation

Histological processing

Mouse handling and dissections

Research Interests

Innate immunology and inflammation

Lung physiology and pathology